# ABUNDANCE AND COMPOSITION OF HETEROTROPHIC PROKARYOTES IN BALLAST WATER DISCHARGED INTO CANADIAN HARBORS

BEI SUN

# Abundance and composition of heterotrophic prokaryotes in ballast water discharged into Canadian harbors

by

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## Abstract

Bacterial abundance and size was characterized in ballast and port water along the West and East coasts of Canada and on the Great Lakes during 2007 and 2008. Ballast water unexchanged at sea showed higher bacterial abundance than ballast water exchanged. On average, bacterial abundance in receiving port water was three-to-four-fold higher than that in ballast water. During 2007, bacterial community structure, as determined by fluorescence *in situ* hybridization, showed that the bacterial communities did not differ among ballast waters which had experienced different ballast operations. However, bacterial communities differed between ballast and port water, which implies that there is a potentially environmental risk from ballast water-distributed bacteria into Canadian harbors. With an increase of ballast water age, both the % *Bacteria* and the % Alpha-Proteobacteria in ballast water samples decreased.

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## List of Abbreviations and Symbols

| | |
|---|---|
| AO | Acridine orange |
| CAISN | Canadian Aquatic Invasive Species Network |
| DAPI | 4',6-diamidino-2-phenylindole |
| EC | East Coast |
| FISH | Fluorescence *in situ* hybridization |
| GL | Great Lakes |
| ICE | Intra-coastal exchange |
| MOE | Mid-ocean exchange |
| NonMOE | Without mid-ocean exchange |
| TOE | Trans-oceanic exchange |
| WC | West Coast |

## Co-Authorship Statement

The research described in this thesis was carried out by Bei Sun, with guidance from Richard B. Rivkin, David C. Schneider and Andrew Lang. Bei Sun was responsible for data collection and analysis. Manuscripts resulting from this thesis were prepared by Bei Sun, with editing assistance and intellectual input from co-authors as follows:

Authorship for publication arising from **Chapter 2** will be Bei Sun, Rebecca Mouland, Candice Way, and Richard B. Rivkin.

Authorship for publications arising from **Chapter 3** will be Bei Sun, Candice Way, and Richard B. Rivkin.

# Chapter 1 Introduction

Aquatic invasive species potentially have adverse affects on biodiversity, public health, and aquaculture as causing tremendous ecological and economic harm (Pimentel et al. 2005). Annually environmental damage and economic losses in the United States alone are valued at an excess of US$137 billion caused by both aquatic and terrestrial alien-invasive species (Pimentel et al. 2005). The increasing global occurrence of invasive species over the last decades has stimulated the establishment of national and worldwide research networks, including the Canada Aquatic Invasive Species Network (CAISN), which studies global aquatic invasions and constructs invasive species profiles (Ruiz et al. 2000).

Heterotrophic prokaryotes (hereafter bacteria) have obtained much attention in aquatic invasion studies after findings showed ships could globally distribute the pathogen *Vibrio cholerae* O1 and O139 (Ruiz et al. 2000). In 1991, ballast water containing the microbe *Vibrio cholerae* was released in a Peruvian port, contaminating the drinking water (Bright 1998). The contamination caused over one million people to be infected with cholera and over 10 000 deaths (Bright 1998). The global distribution of bacteria in ballast water, not only imposes a health risk by spreading epidemic diseases, but can also impact the bacterial communities and the ecological functions of receiving waters.

## 1.1 Roles of Bacteria in Ocean Biogeochemistry

Over the past 3.5 billion years, marine microbes have shaped and defined Earth's biosphere and have created an environment that have allowed the evolution of macroorganisms and complex biological communities (Karl 2007). Bacteria not only constitute a significant portion of the total biomass of the oceans (Whitman et al 1998), but they also have important roles in ocean biogeochemistry (Karl 2007). Bacteria can harvest and transduce solar energy (Grigorieff et al. 1996; Beja et al. 2000), transform dissolved organic matter and recover fixed carbon into microbial food web (Sherr and Sherr 2000), catalyze key biogeochemical transformations of the nutrients and trace elements that sustain the organic productivity of the oceans, and regulate the flow of most greenhouse gases between the ocean and atmosphere (Kirchman 2000; Karl 2007).

### 1.1.1 Dynamics of Bacterial Communities

Some microorganisms have ubiquitous distribution (Finlay 2002), but it does not mean microbial community assemblages from various sites are the same. Open-ocean ecosystems are time-and-space variable mosaics with regards to microbial composition and metabolism (del Giorgio and Bouvier 2002; Cottrell and Kirchman 2003; Smith and del Giorgio 2003).

Bacterial communities are controlled both by "bottom-up" effects, i.e. bacterial food resources, and by "top-down" effects, i.e. the mortality exerted by bacterial predators or viruses (Billen et al 1990; Weisse 1991; Pace and Cole 1994). Further, the growth of

bacteria is influenced by various physiochemical factors, including temperature (Kirchman 1997), salinity (Bouvier and del Giorgio 2002), pH (Hiorns et al. 1997), radiation (Herndl et al. 1993), and trace metal supply (Tortell et al. 1996).

### 1.1.2 Microbial Community Structure and Function

The relationship between bacterial community composition and community ecological functions is complex. Not all phylogenetic groups of bacteria can mediate all elemental processes and transformations, nor do all groups with the same potential metabolic capacities play equal roles. The biogeochemical implications of the phylogenetic diversity of marine bacterial communities are poorly understood (Riemann et al. 1999). For example, *Roseobacter* (a lineage of Alpha-Proteobacteria) is recognized as a major player in the sulfur carrier dimethylsulphoniopropionate (DMSP) turnover within the microbial food web (Zubkov et al. 2001), although Simó (2004) reported *Roseobacter* was not the only player in DMSP cycling. In addition, community functions are not only dependent on components of a community, but are also dependent on the physiological state of cells. Thus, to fully understand the roles prokaryotes play in ocean biogeochemistry, one needs to first determine bacterial abundance, phylogenetic diversity and bacterial metabolic rates (Kirchman 2002).

## 1.2 Global Dissemination of Aquatic Species

As early as 1934, Baas Becking (1934) had referred to atmospheric transport as the major medium to transport microbial "germs" passively over long distances and distribute them

homogeneously over the world. Now, it is recognized that major contributors to bacterial cosmopolitan distribution in marine environments are ocean circulation and shipping. Ships have been identified as the predominant vector for human transport of non-indigenous species around the world (Carlton 1985; Ruiz et al. 2000). A ship may be viewed as a "biological island" with organisms occurring on the outside, inside, and aboard the vessel.

Hull fouling and ballast water are the two primary shipping sub-vectors responsible for coastal invasion (Fofonoff et al 2003). These sub-vectors are responsible for 95% of invasions introduced solely by ships in coastal marine environments off continental North America since 1990 (Fofonoff et al. 2003). Due to changes in shipping regulations, such as increased vessel speeds, decreased port residency, increased use and efficacy of toxic antifouling paints, and an increased frequency of hull cleaning, there have been reductions in the amount of invasive species introduced via ship fouling (Carlton 1985; Carlton et al. 1995). However, for ships to operate safely, ballast water is required. Therefore, ballast water can be seen as a "guaranteed" release of inoculation with scores or hundreds of species, and sometimes million of individuals. Ships can transport ballast water across the ocean, within weeks or months (Smith et al. 1999). Over 80% of the world cargo is mobilized by sea and over 12 billion metric tons of ballast water is filled at one part of the ocean and discharged in another (Anil et al. 2002). Approximately 3 to 5 billion metric tons of ballast water is transferred among international waters annually (Raaymakers 2002). A survey carried out by Canadian Aquatic Invasive Species Network

(CAISN) showed that 4.07 x $10^7$ metric tons ballast water was deballasted into Canadian harbors internationally in 2007 (Lo et al. 2008).

In general, the transfer rate of marine organisms is thought to have increased, especially during the twentieth century, due to changes in the size, speed, and operation of global shipping traffic (Carlton 1996; Ruiz et al. 1997). Additionally, almost all marine plankton, including fouling organisms and benthos with a planktonic larval phase or a semipelagic mode of life, have the potential to be transported by ballast water. Ballast water transfers aquatic organisms to foreign regions. If ballast water is discharged at a habitable environment, surviving plankton will have the opportunity to establish themselves, propagate and potentially displace native species. Therefore, ballast water is one of the primary mechanisms to globally disseminate invasive aquatic species.

## 1.3 Ballast Water Mediated Invasion

Ballast water has been used widely to maintain ship stability and trim during voyages since the early 1880's (Carlton 1985). Typically, as cargo is loaded onboard the ship, the ballast water from the departure port is discharged at the port of call. A successful ballast-mediated invasion is a multistep process whereby the transport of microorganisms in ballast water to new environment is governed by a series of selection processes (Carlton 1985). Firstly, planktonic communities in source water are pumped into ballast tanks. Secondly, the specific environment in ballast water tanks may drive the succession of the biological communities. Lastly, the surviving biota in the ballast-water at the end of

the voyage is released into the receiving water port and selected upon by the suite of environmental conditions there (Carlton 1985).

Increasing awareness about the potentially disastrous consequences of aquatic invasions, has resulted in a surge of additional research focused on a species inventory, primarily the planktonic community present in ballast water (Wonham et al. 2000, 2001; Choi et al. 2005). It is well established that ballast water can contain a diversity of metazoan (Lavoie et al. 1999) and protozoan taxa (Galil and Hülsmann 1997), phytoplankton and their cysts (Hallegraeff and Bolch 1992), and bacteria (Ruiz et al. 2000; Drake et al. 2001). Several well-known instances of ballast-water introductions are the zebra mussel (*Dreissena polymorpha*) to the Great Lakes (Griffiths et al. 1991) and Chinese mitten crab (*Eriocheir sinensis*) to North America and Europe (Herborg et al. 2003).

### 1.3.1 Measures to Attenuate Ballast Water Introduced Invasion

Various ballast water treatments have been attempted to reduce the risk of invasive species from deballasted water. Strategies include biocides (Gregg and Hallegraeff, 2007), filtration, thermal treatment, electric pulse/pulse plasma treatment, ultraviolet, acoustics, magnetic, and deoxygenation (Hallegraeff 1998). However, the application of these control methods are limited by safety requirements, environmental acceptability, technical feasibility and practicability, and cost effectiveness (Tamburrie et al. 2003). Nowadays, the most practical method for ballast water management is ballast water mid-ocean exchange (MOE) (Carlton et al. 1995; International Maritime Organization 1998;

American Bureau of Shipping 1999). Canada initiated mandatory MOE for ocean-going ships entering all Canadian ports in 2006 following the International Maritime Organization guidelines (Transport Canada 2006). However, voyages from certain nearby US ports to Canada do not require MOE as nearby ports would have similar community compositions. Ballast water on board can vary from $5x10^2$ to $8x10^4$ metric tons, depending on the ship types, ship size, and the onboard cargo weight. Ballast water exchange can be accomplished by either the sequential empty-refill method or by flow through. Empty-refill exchange involves emptying the ballast tank completely of port water before uploading ocean water (100%). Flow through exchange involves simultaneously uploading ocean water while allowing excess ballast water to overflow on to the deck. Due to the mixing of ocean and port water during this process, three tank-volumes of water (300%) are required theoretically to remove >95% of the original water (International Maritime Organization, 2004).

Mid-ocean exchange management of ballast water from commercial ships can be divided into transoceanic navigation and intra-coastal navigation. Transoceanic ships are required to exchange their ballast water at more than 200 nautical miles from shore where the water depth is at least 2000 m, whereas intra-coastal ships are required to exchange ballast water at least 50 nautical miles from shore and at water depths of at least 500 m (Transport Canada 2006). Ballast water from arriving international ships falls under one of the three categories: trans-oceanic exchanged (TOE); intra-coastal exchanged (ICE); without mid-ocean exchange (NonMOE).

The rationale for MOE to reduce the nonindigenous species is that the difference in environmental conditions (e.g. salinity, temperature, nutrient richness) between coastal water and the open ocean are inhospitable for the successive survival and propagation of aquatic organisms from foreign regions (Smith et al. 1999). However, this justification may not apply to microorganisms able to tolerate a wide range of environment conditions.

### 1.3.2 Ballast Water Globally Disperses Bacteria

Bacteria possess the ability to invade new environments and are numerically dominant in seawater, occurring in densities of $10^7$ to $10^{10}$ cells $L^{-1}$ (Whitman et al. 1998). Combined with their small cell size (0.2-0.8 μm is the operational definition of bacterioplankton), bacteria are easily transported globally in high abundance. Previous studies have reported that the bacterial abundance in ballast water can range from $10^7$ to $10^9$ cells $L^{-1}$ (Drake et al. 2001, 2002). Furthermore, bacteria can employ a variety of survival strategies, including the formation of spores that enable them to withstand prolonged periods of inhospitable conditions (Karl 2007). In addition, short generation time (Riemann et al. 1987) enables bacteria to quickly populate new environment.

Strong bias exists for the studies of taxonomic invasions caused by ballast water, and the prevalence of small organisms (e.g. bacteria) is grossly underrepresented by current studies (Ruiz et al. 2000). The available baseline information for bacteria transported in ballast-water is poorly established (Elbraechter 1999). Prior studies about bacteria in ballast water have generally been limited to bacterial abundances (Drake et al. 2001, 2002)

and the occurrence of pathogens (Ruiz et al. 2000; Burkholder et al. 2007). Global movement of ballast water by ships creates a long-distance dispersal mechanism for human pathogens and waterborne diseases affecting plants and animals (Ruiz et al. 2000). Also, the global transport of species brings together pathogens and previously unexposed host populations (Harvell et al. 1999). Although total bacterial abundance and occurrence of pathogens is informative, further studies on phylogenetic group composition of introduced communities (Carlton 1996) are needed to predict species invasion of marine environments.

Bacterial communities in ballast water tanks are influenced by myriads of factors, among which, ballast water operation (i.e. if MOE, exchange location) (Wonham et al. 2001), "bottom-up" and "top-down" effects (Drake et al. 2001), temperature, salinity (Riemann and Middelboe 2002), and ballast water age (Burkholder et al. 2007) are main factors. Understanding bacterial dynamics in response to these factors would allow researchers to identify ways to better control invasions as well as methods to better predict and prevent future invasions.

## 1.4 Objectives and Justification of Study

As part of the CASIN, the bacterial abundance and community structure of ballast and receiving port waters were characterized. This research was undertaken to assess the potential impacts posed by ballast water introduced bacteria on the Canadian aquatic environment. Sampling was carried out between March 2007 and November 2007, and

between May 2008 and October 2008. Samples were collected along the West Coast (Vancouver, British Columbia) and East Coast (2007: Baie-Comeau, Sept-Iles, Port-Cartier, along the lower north shore of the St Lawrence Estuary, Quebec; 2008: Saint John, New Brunswick; Point Tupper, New Brunswick; Halifax, Nova Scotia; Auld Cove, Nova Scotia; Corner Brook, Newfoundland and Labrador) of Canada, and the Great Lakes (Toledo, Ohio, USA; Milwaukee, Wisconsin, USA; Detroit, Michigan, USA; and Sarnia, Ontario, Canada). Environmental parameters (temperature, pH, and salinity) and ballast water reporting forms (ballast water source, if MOE conducted, mid-ocean exchange locations, voyage duration, and ballast water age) were also recorded.

This thesis has been organized into two manuscript chapters:

Chapter 2 (*Bacterial abundances and cell volumes in ballast water discharged into Canadian harbors*) examines the propagule pressure of ballast water bacteria conveyed to Canadian waters. By comparing the bacterial dynamics (bacterial abundance and cell volume) between ballast water and receiving port water samples, the extent of bacterial propagule pressure from ballast water to receiving port water was estimated. In addition, the relationships of measured bacterial variables (bacterial abundance and cell volume) among the three ballast water types (ICE, TOE and NonMOE) were studied to evaluate the effect of different ballast water operations (ICE, TOE and NonMOE) on bacteria in ballast waters. Lastly, the relationships between measured bacterial variables (bacterial abundance and cell volume) and physiochemical factors (e. g. temperature, salinity, pH,

ballast water age, and exchange locations) were also investigated to identify potential ballast water treatment measurement.

Information about bacterial community composition, in addition to simple abundance, is needed to evaluate the disturbance to receiving waters from bacteria introduced from ballast water. Chapter 3 (*Bacterioplankton communities distributed globally by ballast water*) investigated the prokaryotic community structure using fluorescence *in situ* hybridization (FISH). The proportion of ubiquitous phylogenetic groups (e.g. *Bacteria, Archaea,* Alpha-Proteobacteria, *Cytophaga-Flavobacteria*) and potential pathogens (e.g. *Vibrio* spp., *Escherichia coli*) within bacterial communities were assessed. The objectives of this chapter were to evaluate different ballast water operation effects (ICE, TOE and NonMOE) on bacterial communities in ballast waters, and to assess the differences in bacterial community structure between ballast water and receiving ports in the three regions. The relationships between bacterial community composition and environmental factors (i.e. temperature, salinity, pH, ballast water age, exchange locations) were also investigated.

The results of this study are an important contribution to our knowledge of the abundance, cell volume, and phylogenetic diversity of the bacterial community in ballast water tank on large spatial and temporal scales. The study will also give some insight about the effects of ballast water management applications in Canada to the bacterial communities in ballast water.

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# Chapter 2 Bacterial Abundances and Cell Volumes in Ballast Water Discharged into Canadian Harbors

## 2.1 Abstract

Ballast water discharged by oceangoing ships contributes to the introduction of benign and pathogenic invasive species. As part of the Canadian Aquatic Invasive Species Network, we studied the abundance and cell sizes of heterotrophic prokaryotes (hereafter bacteria) in ballast and receiving port waters. The study was carried out from March to November 2007, and May to October 2008, with samples collected along the West and East coasts of Canada and on the Great Lakes. Bacterial abundance in port water was three-to-four-fold higher than in ballast water. Among the ballast water types, unexchanged ballast water showed higher bacterial abundance than ballast water exchanged at-sea. There was no significant difference in bacterial abundance between 2007 and 2008 for each ballast water sample type and for port samples. There was no significant difference in average bacterial cell volume between ballast water and port water samples; however the average bacterial cell volumes were about two-fold larger during 2007 than 2008. The average bacterial abundance and cell volume in ballast water did not show specific regional difference among the Great Lakes, the West and East coasts of Canada and were generally unrelated to ballast water age, end-of-voyage temperature, salinity and pH. The exception was a negative relationship between the bacterial abundance of intra-coastal exchanged samples and ballast water age. We estimated that $3.3x10^{19}$ prokaryotic cells are transported into Canadian ports annually by

ballast water. Although bacterial abundances in ballast waters were lower than in receiving harbors, future studies should focus on the bacterial community structure in ballast and ports waters, the fate of bacteria in the new environment and regional susceptibility to invasion by the introduced bacteria.

## 2.2 Introduction

Many microorganisms (prokaryotes and microbial eukaryotes) are abundant, fast growing, tolerant to a wide range of environments and easily dispersed, hence there are few geographic barriers to their dispersion (Finlay 2002). For small-sized microscopic organisms, distribution is mediated by a range of passive mechanisms, such as ocean currents, sea spray and aerial transport, and migrating organisms (Fenchel et al. 1998; Finlay 2002). In marine environments, ocean circulation and shipping are primary processes for transport of microorganisms.

Seawater has been used as ballast to help vessels keep stability and trim during voyages since the late 1870s (Carlton 1985). Over 80% of the world's cargo is mobilized internationally by ships and over 12 billion metric tons of ballast water is filled at one part of the ocean and discharged at the other annually (Anil et al. 2002). Ballast water has been recognized as the primary vector for the transport of aquatic microorganisms (Carlton and Geller 1993; Ruiz et al. 2000), and it has been calculated that about $10^{18}$ viable bacteria are transported between continents annually by this passive mechanism (Fenchel and Finlay 2004).

Successful establishment of nonindigenous organisms can cause unwanted economic (Pimentel et al. 2005), ecological (Mills et al. 1993; Carlton et al. 1990; Frenot et al. 2005) and human health impacts (Hallegraeff 1998; McCarthy and Khambaty 1994; Juliano and Lounibos 2005). Species abundance have shown consistently positive correlations with species establishment success of invasives in introduced environments (Lockwood et al. 2005). Thus, the abundance of introduced undesirable species is a risk-indicator for future invasion (McCarthy and Crowder 2000). To attenuate the risk of ballast water-mediated invasions, the International Maritime Organization (IMO) established mid-ocean ballast water exchange (MOE) guidelines. The ballast water collected from coastal regions is required to be exchanged in open ocean. The rationale for MOE is that most coastal organisms will be flushed out during exchange (being replaced by oceanic species) and that the different physicochemical parameters between coastal and oceanic waters will impair the survival of coastal organisms in the open ocean. Similarly, oceanic species released into ports with coastal environmental conditions will be unlikely to survive or establish themselves (Smith et al. 1999). However, this justification may not be valid for microorganisms with wide environmental tolerances.

Under the IMO ballast water management guidelines, Canada initiated mandatory mid-ocean exchange (MOE) for ocean-going ships entering all Canadian ports as of June 8$^{th}$, 2006 (Transport Canada, 2006). However, limited voyages from certain nearby US ports to Canada have no compulsory MOE requirement, because similar community compositions are expected between the nearby ports. Mid-ocean exchange of ballast

water from commercial ships can be divided into transoceanic and intra-coastal navigation. Transoceanic ships are required to exchange their ballast water more than 200 nautical miles from shore where the water depth is at least 2000 m. In contrast, intracoastal ships are required to exchange ballast water at least 50 nautical miles from shore and at water depth of at least 500 m. Therefore, ballast water from ships arriving from international destinations falls into three categories: trans-oceanic exchanged (TOE); intra-coastal exchanged (ICE); without mid-ocean exchange (NonMOE).

As part of the Canadian Aquatic Invasive Species Network, a study was carried out to examine average bacterial abundances and cell volumes in ballast water (TOE, ICE, NonMOE) and receiving port water for ships that deballasted into different areas (the Great Lakes, the West Coast and East Coast of Canada) during 2007 and 2008. The objectives of this study are to evaluate different ballast water exchange protocols (ICE, TOE and NonMOE) on measured biological variables (bacterial abundance and cell volume) in ballast waters; to assess the differences in measured biological variables between ballast water and receiving ports in the three regions; to assess if there are differences in ballast water bacteria discharged into the three sampling regions; and to assess the relationships between measured biological variables (bacterial abundances and cell volumes) and concurrent physiochemical factors (i.e. temperature, salinity, pH, and ballast water age).

## 2.3 Materials and Methods

### 2.3.1 Fixative Effects on Bacterial Abundances and Cell Volumes

Both glutaraldehyde and formaldehyde (final concentration vol/vol 2%) are routinely used to preserve samples for the determination of bacterial abundance using acridine orange (AO) staining (Turley and Hughes 1992). Formaldehyde (final concentration vol/vol 3.7%) is also used to preserve samples for analysis of community structure by fluorescence *in situ* hybridization (FISH) (Glöckner et al. 1996). In this study, samples will be used for both bacterial abundance study with AO staining (Chapter 2) and community structure study with FISH (Chapter 3). The suitability of formaldehyde (final concentration vol/vol 3.7%) for acridine orange direct count (AODC) method was investigated in this study.

A five-liter seawater sample was collected from Logy Bay, Newfoundland, Canada (47°37'30.32"N, 52°39'48.36"W) on January 3rd, 2007. Replicate 500ml samples were preserved with 0.2 μm filtered glutaraldehyde (final concentration vol/vol 2%). Another replicate of 500ml samples were preserved with 0.2 μm filtered formaldehyde (final concentration vol/vol 3.7%). Preserved samples were stored at 4°C, and sub-samples were taken at 2 h, 1 d, 2 d, 3 d, and 5 d. Samples were filtered onto 25mm diameter, 0.2 μm black polycarbonate filters (GE Osmonics Labstore, Minnetonka), stained with AO (final concentration $1.872\times10^{-5}$g $L^{-1}$) (Hobbie et al. 1977; Kirchman et al. 1982). Slides were corrected for non-cell staining by counting of 5 ml of 0.2 μm filtered distilled water

before each sample filtration. To eliminate possible bias introduced by prolonged slide storage, all slides were counted within one hour after being made.

### 2.3.2 Preservation Duration Effects on Bacterial Abundances and Cell Volumes

Turley and Hughes (1992) have reported an average 39% bacterial cell loss after 40 days storage in 2% glutaraldehyde suspension. Therefore, they recommended that collected water samples should be fixed, stained, and then slides prepared as soon as possible after sample collection. However, it could take days to transport water samples from sampling sites to our laboratory for filtration. To make sure there is comparability among samples with different preservation duration (time between fixative addition and filtration), short-term (five days) preservation effects on bacterial abundance and cell volume were tested in this study.

A 10-liter seawater sample was collected from Logy Bay, Newfoundland, Canada (47°37'30.32"N, 52°39'48.36"W), on March 13th, 2007. Five replicate 1L sub-samples were preserved with formaldehyde (final concentration vol/vol 3.7%). Samples were stored at 4 °C, and sub-sampled at 2 h, 1 d, 2 d, 3 d, and 5 d. Slides were prepared as described above.

### 2.3.3 Ballast Water and Port Water Sampling

Sampling was carried out from March to November 2007, and May to October 2008 from three sampling regions of Canada (EC-East Coast; GL-Great Lakes; WC-West Coast) that actively engage in international shipping. These site included the West Coast (Vancouver, British Columbia) and the East Coast of Canada (2007: Baie-Comeau, Sept-Iles, Port-Cartier, all on the lower north shore of the St Lawrence Estuary, Quebec; 2008: Saint John, New Brunswick; Point Tupper, New Brunswick; Halifax, Nova Scotia; Auld Cove, Nova Scotia; Corner Brook, Newfoundland and Labrador), and the Great Lakes (Toledo, Ohio, USA; Milwaukee, Wisconsin, USA; Detroit, Michigan, USA; and Sarnia, Ontario, Canada). Commercial vessels which arrived at those ports and had ballast tanks with TOE, ICE, and NonMOE, were sampled. Port water samples were periodically collected during sampling seasons.

After onboard each vessel, CAISN sampling teams sampled one or two ballast tanks per ship. For each ballast tank, samples were collected, through a deck hatch, by a Niskin bottle lowered to four depths in the tank (surface, mid surface, mid bottom, and bottom). Equal volumes of ballast water from each of the sampling depths were combined together from the same ballast tank. Associated environmental data (temperature, salinity, and pH) was also recorded from each sampling depth using a handheld YSI Model 85 meter equipped with a 15 m cable (YSI Incorporated, Yellow Springs, OH, USA). A sub-sample (500ml) of the combined ballast water from one tank was preserved with formaldehyde (final concentration 3.7%) and shipped on ice to the Ocean Sciences Centre (St. John's,

Newfoundland and Labrador, Canada) for analyses within five days. Along with the collected ballast water samples, the ballast water management forms with information on vessel type, ballast water source, exchange location, ballast water deballast port, onboard ballast water volume, exchanged ballast water volume and deballast water volume of sampled vessels data were provided.

### 2.3.4 Epifluorescence Microscopy

The slides for bacterial abundances and cell volume measurements were observed using an Olympus BH2-RFC epifluorescence microscope, equipped with a 100 x 1.30 oil objective (1250 x total magnification), a 100 W mercury lamp and appropriate filter sets (502 nm for excitation, 526 nm for emission). The optimal cell direct counting scheme was followed to optimize the accuracy of the direct count (Kirchman 1982). Briefly, for sample slides, 50 boxes out of 100 boxes in an ocular grid were counted per field, 10 to 20 random selected fields were scanned each slides until more than 600 cells were counted from one filter. For the determination of background counts, we counted 100 boxes per field and 10 fields were scanned per slide.

### 2.3.5 Image Analysis

Cell dimensions were determined with an Image-Pro Plus V6.2 Image Analysis System (Media Cybernetics, Inc., Bethesda). The system is configured to capture and store images, measurements and size distributions. In this image analysis study the epifluorescence microscope was equipped with a highly sensitive camera linked to a

desktop computer. The image analysis system used was digitally calibrated using a stage micrometer and initial calibration software. Images of cells which fluoresce on the slide were captured by the camera and the image diverted to a Dell PC. The cell diameters were measured: cell lengths and cell widths. The images were individually examined, and the cell dimensions were recorded. Manual determination of the fluorescence intensity threshold was essential in determination of cell edge locations. Detrital particles or specific cells (i.e. clumped or aggregated) were screened out from the analysis either through the direct removal from the working image, or by constraints assigned to acceptable diameters. Slides for individual experiments were made at the same time so that bacterial slide quality and cell sizes within an experiment were comparable. The output measured variables for each sample were diverted to an Excel spreadsheet. Cell volumes were calculated using formulae created for volume determination of spheres or cylinders. Cells with an aspect ratio <1.5 were calculated as spheres whereas cells with an aspect ratio >1.5 were calculated using the formula for cylinders. Mean cell volume was determined for each shape class (round-shaped and rod-shaped cells).

### 2.3.6 Statistical Analyses

The effects of the glutaraldehyde and formaldehyde fixatives at each of the preservation durations (2 h, 1 d, 2 d, 3 d, and 5 d) on the average bacterial abundance and cell volume of samples were analyzed by paired *t*-test. Regressions were applied to estimate the relationship between preservation duration and bacterial parameters (abundance and cell volume). Multivariate Analyses of Variance (ANOVA) were applied first to analyze the

relationships of measured bacterial parameters among different sample types, sampling locations, and different years; however, the statistics showed that the interaction terms were significant. Therefore, one-way ANOVA were carried out to compare the measured bacterial parameters (bacterial abundances and cell volumes) among the three ballast water types, and between ballast and port waters at each deballasting location, among three deballasting locations, and between sampling years (2007 and 2008). If a statistically significant result was found in an omnibus F-test for a one-way ANOVA, post-hoc analyses using the Tukey test were conducted. Regression analyses were run to determine the relationships between bacterial abundances and environmental variables (i.e. temperature, pH, salinity, ballast water age), and relationships between cell volumes and these environmental variables.

All statistical analyses were conducted using Minitab Release 14 (Minitab Inc., State College, USA). For each analysis, the residuals were examined and met the assumptions of linearity, normality, independence, and homogeneity (Seber and Lee 2003). The significance judge criterion for statistics in this study is $\alpha = 0.05$.

## 2.4 Results

### 2.4.1 Fixative Effects on Bacterial Abundances and Cell Volumes

There were no significant differences in bacterial abundances and cell volumes for the two fixatives at each of the storage times ($n = 20$; bacterial abundance: $t = 0.19$, $P = 0.856$; cell volume: $t = 2.61$, $P = 0.060$). However, we did observe better contrast between the

AO stained cells and background fluorescence for formaldehyde than glutaraldehyde preserved samples. Therefore, subsequent samples for bacterial abundances, cell volumes and bacterial community structure investigations were preserved by 0.2 μm filtered formaldehyde (final concentration vol/vol 3.7%).

### 2.4.2 The Effects of Preservation on Bacterial Abundances and Cell Volumes

Regressions were used to determine if bacterial abundances and cell volumes were preservation-duration dependent. Within 5 days preservation in suspension, there was no significant difference in bacterial abundances ($n = 25$, $P = 0.191$), whereas cell volumes increased with the preservation time following an exponential growth rate: Cell Volume = $0.0731 + 0.0030$ Duration, $r^2 = 0.52$, $n = 25$, $P < 0.001$ (Figure 2.1).

### 2.4.3 Vessels Sampled and Ballast Water Management

A total of 139 ballast water samples (NonMOE, ICE, and TOE) and 13 port water samples were collected from March to November 2007, and May to October 2008. Table 2.1 summarizes the sample number in each group defined by sampling year (2007, 2008), sampling locations (Great Lakes, West Coast and East Coast of Canada), and sample types (TOE, ICE, NonMOE, port).

This study primarily included commercial bulk carriers, general cargo, and tankers (91% of total ballast water samples or 126 of 139 ballast water samples), with a few containers,

roll on/roll off and multi-purpose ships. The pie graph in Figure 2.2 shows the composition of ballast water sample by vessel types (bulk carriers, general cargo vessels, tankers and others) at WC, EC, and GL during 2007 and 2008 sampling seasons. The majority of ballast water collected from the WC was from bulk carriers and general cargo vessels, while the majority of ballast water samples from GL and the EC were from bulkers and tankers.

### 2.4.4 Ballast Water Ages and Physical/Chemical Characteristics of Collected Samples

Ballast water ages were computed as the number of days between the date of exchange for MOE (ICE and TOE) or take-up in the port for NonMOE samples and sampling date. The mean (± SD) ballast water age of ICE samples was (6.7 ± 6.6) days (median = 3.5, n = 56), which was shorter than that of TOE samples (10.7 ± 5.7) days (median = 10.5, n = 54). NonMOE samples (mean ± SD = 7.4 ± 17.0, median = 3.5, n = 28) had the oldest ballast water, ranging from 1-93 days, compared to 1-27 days for ICE and3-43 days for TOE.

Physical/chemical parameters of all tanks showed no depth stratification in pH, salinity, and temperature (data not shown). The average of the four sampling depths recorded for each ballast water parameter are presented. Temperature of port water samples ranged from 1°C in May 2007 at the EC to 26°C in August 2007 and August 2008 at the GL. Temperature of ballast water samples ranged from 3.6°C in May 2007 at the EC to 30.6

°C in August 2008 at the EC. Temperature of port, TOE, ICE, NonMOE samples all followed the same monthly patterns during the two sampling seasons (April to November in 2007; May to October in 2008) (Figure 2.3). The mean temperature of samples increased starting in spring, peaked at end of summer in August, and then started to decrease (Figure 2.3). Salinity showed distinct characteristics for oceanic (TOE and ICE) and coastal (port and NonMOE) source samples. Oceanic source samples had higher salinity with narrow range: salinities of TOE water samples ranged from 24.7 to 37.0 psu (practical salinity units) (mean 33.6 psu); salinity of ICE water samples ranged from 25.8 to 36.1 psu (mean 32.2 psu). In contrast, coastal sources samples had wide salinity range: salinity of port water samples ranged from 0.0 psu at the GL to 32.4 psu at the EC; salinity of NonMOE water samples ranged from 0.1 to 31.2 psu. The pH ranged from 6 to 8.5.

### 2.4.5 Heterotrophic Bacterial Abundance

### Comparison among Ballast Water Types

The bacterial abundances among ballast water types (TOE, ICE and NonMOE) at each sampling region during 2007 and 2008 are reported in Table 2.2. There was no significant difference in the bacterial abundance between TOE and ICE samples at the EC ($F_{1, 21} = 0.47$, $P = 0.503$, Table 2.2) and GL ($F_{1, 9} = 0.04$, $P = 0.852$, Table 2.2). The number of NonMOE samples collected at the EC and GL was too low for statistical analysis. ANOVA results showed that there was a significant difference in bacterial abundance among TOE, ICE and NonMOE at the WC in 2007 ($F_{2, 25} = 3.50$, $P = 0.046$, Table 2.2).

Post-hoc comparisons using the Tukey test indicated that bacterial abundance was significantly higher in NonMOE than TOE samples, but there was no significant difference between NonMOE and ICE, or between TOE and ICE samples at the WC site. In 2008, there was no significant difference in bacterial abundance between ICE and NonMOE samples at the EC ($F_{1, 23} = 1.24$, $P = 0.276$, Table 2.2). The number of TOE samples collected at the EC was too low for statistical analysis. Only TOE samples were collected at the GL in 2008, so no comparison among ballast water types was conducted. ANOVA results showed that there was a significant difference in bacterial abundance among TOE, ICE and NonMOE at the WC in 2008 ($F_{2, 38} = 4.18$, $P = 0.023$, Table 2.2). Post-hoc comparisons using the Tukey test indicated that bacterial abundance was significantly higher in NonMOE than TOE samples, but there was no significant difference between NonMOE and ICE, or between TOE and ICE samples at the WC. Taken together, the bacterial abundance was significantly higher in NonMOE than TOE samples, but there was no difference detected in bacterial abundance between TOE and ICE samples, or between NonMOE and ICE samples.

## Comparison between Port and Ballast Water Samples

The bacterial abundance in port water samples ranged from $3.44 \times 10^8$ to $1.01 \times 10^{10}$ cells $L^{-1}$ and was three-or-four-fold higher than that in ballast water samples which ranged from $8.69 \times 10^7$ to $2.60 \times 10^9$ cells $L^{-1}$. The bacterial abundance of ballast water and port water samples in each region each year is shown in Figure 2.4. Although bacterial abundance was generally higher in port water than ballast water samples at each

deballasting location in both years, the statistical differences were detected only at the WC in 2007 ($F_{1, 29} = 28.59$, $P < 0.001$, Table 2.4), the EC ($F_{1, 36} = 13.11$, $P = 0.001$, Table 2.4) and WC ($F_{1, 49} = 40.15$, $P < 0.001$, Table 2.4) in 2008. Too few port water samples were collected from the GL in both years to compare bacterial abundance between ballast water and port water samples in the GL.

## Comparison among Deballasting Locations

The limited number of port water samples collected from the GL in both years prevented their inclusion in the port water samples comparison. The bacterial abundance of port water samples was significantly higher in the WC than in the EC in 2007 ($F_{1, 6} = 7.43$, $P = 0.034$, Table 2.4), but no significant difference was detected between the WC and EC port water samples in 2008 ($F_{1, 19} = 1.53$, $P = 0.232$, Table 2.4).

There was no significant difference in the bacterial abundance of TOE samples among the three deballasting locations ($F_{2, 38} = 0.66$, $P = 0.524$, Table 2.2); neither for ICE samples ($F_{2, 21} = 0.71$, $P = 0.510$, Table 2.2) during 2007. Too few NonMOE samples were collected from the EC and GL for statistical comparisons during 2007 (Table 2.2). During 2008, bacterial abundances in TOE samples were compared between the GL and WC only, since the number of TOE samples collected at the EC was too low for statistical analysis. There was no significant difference in bacterial abundance of TOE samples between the GL and the WC during 2008 ($F_{1, 17} = 1.93$, $P = 0.182$, Table 2.2). There were no ICE and NonMOE samples collected from the GL, so the bacterial abundances in the ICE and

NonMOE samples were compared between location EC and WC in 2008. During 2008, the bacterial abundances were higher at the EC than at the WC for both ICE ($F_{1, 31} = 6.71$, $P = 0.014$, Table 2.2) and NonMOE samples ($F_{1, 20} = 8.52$, $P = 0.008$, Table 2.2). In general, ANOVA results showed that the bacterial abundance in ballast water that was deballasted into the three regions was significantly different in 2008 ($F_{2, 73} = 14.18$, $P < 0.001$, Table 2.4). Post-hoc comparisons using the Tukey test indicated that the bacterial abundance in ballast water that was deballasted into the EC was higher than into the WC and the GL during 2008. However, there was no significant difference in the bacterial abundances of ballast water samples that were deballasted into the WC and GL sites.

### Comparison between Years

Due to low and variable sample sizes, comparison of bacterial abundances between years was only tested for port, ICE samples at the EC; TOE samples at the GL; and port, TOE, ICE and NonMOE samples at the WC. There were no significant differences in bacterial abundances between 2007 and 2008 for any above comparisons (Table 2.3, Table 2.2).

## 2.4.6 Cell Volume

### Comparison among Ballast Water Types

The cell volumes for the different ballast water types in each sampling region during 2007 and 2008 are reported in Table 2.2. There was no significant difference in the cell volume between TOE and ICE samples at the EC ($F_{1, 21} = 0.01$, $P = 0.931$, Table 2.2) or GL ($F_{1, 9} = 0.13$, $P = 0.724$, Table 2.2). The number of NonMOE samples collected at the EC and

GL was too low for statistical analysis. ANOVA results showed that there was significant difference in cell volume among TOE, ICE and NonMOE at the WC in 2007 ($F_{2, 25} = 4.20$, $P = 0.027$, Table 2.2). Post-hoc comparisons using the Tukey test showed that the cell volume was significantly larger in ICE samples than TOE samples at the WC in 2007, but there was no significant difference in cell volume between TOE and NonMOE samples, or between ICE and NonMOE samples. In 2008, there was no significant difference in cell volume between ICE and NonMOE samples at the EC ($F_{1, 23} = 0.01$, $P = 0.920$, Table 2.2). The number of TOE samples collected at the EC was too low for statistical analysis. Only TOE samples were collected at the GL in 2008, so no comparison among ballast water types was conducted. ANOVA results show that there was significant difference in cell volume among TOE, ICE and NonMOE samples at the WC in 2008 ($F_{2, 38} = 4.47$, $P = 0.0183$, Table 2.2). Post-hoc comparisons using the Tukey test indicated that cell volume was significant larger in TOE than NonMOE samples, but there was no significant difference between NonMOE and ICE, or between TOE and ICE samples at the WC site in 2008.

## Comparison between Port and Ballast Water Samples

The cell volume comparisons between ballast water and port water samples at each location site each year are shown in Table 2.4. The cell volumes in port water samples was significantly larger than those of ballast water samples at the EC in 2007 ($F_{1, 26} = 5.13$, $P = 0.032$, Table 2.4). This was the only detected significant difference in cell

volume between port water and ballast water samples among all the comparisons in Table 2.4.

## Comparisons among Deballasting Locations

The limited number of port water samples collected from the GL in both years prevents them from being included in the port water samples comparison among deballasting locations (Table 2.1). There was no significant difference in cell volume of port water samples between the WC and EC in both 2007 ($F_{1, 6} = 0.32$, $P = 0.591$, Table 2.4) and 2008 ($F_{1, 19} < 0.01$, $P = 0.990$, Table 2.4).

Too few NonMOE samples were collected from the EC and GL to compare NonMOE samples among locations in 2007. There was no significant difference in cell volume of ICE samples among the three deballasting locations in 2007 ($F_{2, 12} = 2.57$, $P = 0.118$, Table 2.2). ANOVA results showed that there was significant difference in the cell volume of TOE samples among the three regions in 2007 ($F_{2, 38} = 4.62$, $P = 0.016$, Table 2.2). Post-hoc comparisons using the Tukey test indicated that the cell volume of TOE samples was significantly larger at the GL than at the EC and WC in 2007, but there was no difference in cell volume of TOE samples between the EC and WC in 2007. During 2008, cell volume of TOE samples was compared between the GL and WC only, since the number of TOE samples collected at the EC was too low for statistical analysis. There was no significant difference in cell volumes of TOE samples between the GL and WC in 2008 ($F_{1, 17} = 0.47$, $P = 0.502$, Table 2.2). There were no ICE and NonMOE samples

collected from the GL in 2008, so the cell volume of ICE and NonMOE samples were compared between location EC and WC. There was no significant difference in cell volume between the EC and WC for ICE samples ($F_{1, 31} = 0.03$, $P = 0.860$, Table 2.2), or for NonMOE samples ($F_{1, 20} = 0.83$, $P = 0.372$, Table 2.2).

### Comparison between Years

The between-year comparisons of cell volume were tested for port and ICE samples at the EC; TOE samples at the GL; and port, TOE, ICE and NonMOE samples at the WC, due to low sample sizes. Cell volumes were significantly larger for 2007 than 2008 samples in all of the above comparisons (Table 2.3, Table 2.2). Cell volume of all 2007 samples (mean = 0.083 $\mu m^3$; range 0.051 to 0.162 $\mu m^3$; n = 72) was about 1.5-fold larger than during 2008 (mean = 0.055 $\mu m^3$; range 0.039 to 0.088 $\mu m^3$; n = 99).

## 2.4.7 Relationships between Microbial Parameters and Environmental Factors

The relationships between bacterial variables (bacterial abundance and cell volume) of each ballast water type and physiochemical factors (salinity, pH, temperature, and ballast water age) were examined. The cell volume data was divided by year, because of the different cell volume ranges in 2007 and 2008. However, there was no significant difference in bacterial abundance between 2007 and 2008 samples, so the bacterial abundance of samples from both years was analyzed together. There were no significant relationships between bacterial variables and salinity, pH, temperature and ballast water

age, except that the bacterial abundances of ICE samples decreased with the aging of ballast water ($n = 56$, $r^2 = 0.18$, $p < 0.01$, Figure 2.5).

## 2.5 Discussion

### 2.5.1 Fixative Effects on Bacterial Abundances and Cell Volumes

In this study, seawater samples preserved with formaldehyde showed better contrast between the AO fluorochrome stained cells and filter background fluorescence than samples preserved with glutaraldehyde. Formaldehyde has been noted to negatively affect cell fluorescence (Crissman et al. 1978; Lebaron et al. 1998; Troussellier et al. 1999); however, glutaraldehyde frequently leads to intense autofluorescence which limits its use with fluorescent stains (Gasol and del Giorgio 2000; Vives-Rego et al. 2000). There was no significant difference observed in bacterial abundances and cell volumes between glutaraldehyde (final concentration vol/vol 2%) fixed samples and formaldehyde (final concentration vol/vol 3.7%) fixed samples at each of the storage times. The results indicated that formaldehyde (final concentration vol/vol 3.7%) can be used to preserve water samples to study both bacterial abundance and cell volume with AO staining and bacterial community structure with FISH.

### 2.5.2 The Effects of Preservation on Bacterial Abundances and Cell Volumes

Loss of visible (or stainable) bacteria has been noted after months of storage (Turley and Hughes 1992; Gundersen et al. 1996). Two possible non-exclusive explanations for the

cell loss are: (1) bacteria attached to bottle inner surface (Turley and Hughes 1992); (2) bacteria were degraded by enzymatic or viral activity (Gundersen et al. 1996). We did not observe bacterial cell loss following fixation and storage within five days. However, it is worth noting that the relationship between bacterial abundance and preservation duration is sample specific (Gundersen et al. 1996; Turley and Hughes 1992). A similar experiment reported that the concentration of bacteria decreased sharply in the first week of storage in two out of four samples collected from different locations and depths (Decamp and Rajendran 1998).

Cell volume is widely used for bacterial organic carbon (BOC) estimation and to evaluate the bacterial mediated carbon cycle (Simon et al. 1992). The long-term preservation effects of vol/vol 2.0% formaldehyde on cell volumes have been studied, and it was reported that cell volumes significantly increased after 120 days of preservation (Matthews and Rivkin, 2002). In this study, samples preserved with vol/vol 3.7% formaldehyde demonstrated an increase in cell volume during five-day storage at 4 °C. Formaldehyde is a cross-linking fixative which forms DNA-protein cross-links within the cells. It has been reported that AO not only binds to both DNA and RNA, but also stains other structures in the cell (e.g. the cell wall) (Suzuki et al. 1993). The cross-linked structures formed during the storage might cause the observed cell volume increase.

### 2.5.3 Ballast Water Mediated Bacterial Invasion

Bacteria in ballast water at the end of a voyage represent those cells and groups that survived after a series of selections (Carlton 1985). The main selective factors for

surviving bacteria include bacterial communities composition from source waters, the specific environment in ballast water tanks (Carlton 1985) and effects of MOE. The abundance and species diversity in a ballast tank depends very much on the location and seasonality of the water ballasted. Within the tank, the environmental conditions may drive the succession of bacterial communities (Carlton 1985) including absence of light, salinity, pH and temperature fluctuation with the ambient temperature of the ocean (Chu et al. 1997). If the ballast water is exchanged in the mid-ocean, most coastal organisms would be replaced by oceanic organisms and the physiochemical parameters of ballast water would be similar to those of open ocean water where the exchange occurred (Wonham et al. 2001). Coastal environments generally have higher bacterial abundance than oceanic environments. Along a 3 800 km transect from the coastal waters of Monterey Bay, California to the open ocean Hawaii Ocean Times-Series station, the coastal station had the highest bacterial abundance (Culley and Welschmeyer 2002). Similar results have been reported that the bacterial abundance declined progressively with increasing distance from shore (to 100 km) in the euphotic zone of the Southern California Bight, USA (Fuhrman et al. 1980). In addition, bacterial community structures are different between coastal and oceanic environment. Rappe et al. (2000) studied the clone libraries of SSU rRNA genes of seawater collected over the western continental shelf of the USA in the Pacific Ocean. It was concluded that coastal bacterial communities have phylogenetic groups found prevalently distributed both in open ocean and in freshwater (Rappe et al. 2000).

### 2.5.4 Bacterial Abundance Comparison among Ballast Water Types

The number of samples that were collected from each ballast water category (i.e. TOE, ICE, and NonMOE) was sufficient only at the WC to carry out robust statistical analyses among ballast water types. Significantly higher bacterial abundance was observed in NonMOE samples than in TOE samples during both years. Our result indicates that unexchanged ballast water introduces higher propagule pressure than ballast water exchanged on the high seas.

### 2.5.5 Bacterial Abundance Comparison between Port and Ballast

Table 2.5 summarizes published studies about bacteria in ballast water. Bacterial abundances in ballast water samples ranged from $8.69 \times 10^7$ to $2.60 \times 10^9$ cells $L^{-1}$ ($n = 142$) in our study and was similar to reported abundances of previous studies (Table 2.5). Bacterial abundance in port water samples was about three- or four-fold higher than that in ballast water samples in our study. Higher bacterial abundance in receiving ports than that in ballast water was also documented in Singapore (Joachimsthal et al. 2003; 2004), and in Chesapeake Bay (Drake et al. 2001). Ballast water samples studied here were collected at the end of voyage, so both ballast water environment selection and oceanic source for exchanged ballast water samples could be probable reasons for the attenuated bacterial abundance in ballast water samples. The concentration of microorganisms in ballast waters has been considered as a proxy for propagule pressure (the number of individuals introduced into a given environment), which is a prime factor to explain the success of an invasion (Drake and Lodge 2007; Hayes and Barry 2008). Therefore, the

lower bacterial abundance in ballast water observed here suggests that the ballast water operations have attenuated the propagule pressure from the numerical standpoint.

### 2.5.6 Bacterial Abundance Comparison among Deballasting Locations

In a previous study about ballast water discharged along the West and East coasts of USA (Burkholder et al. 2007) showed that bacterial abundance was significantly lower in ballast tanks with Atlantic than Pacific Ocean source water. In our study, ballast waters deballasted into the WC were from the Pacific Ocean, whereas ballast waters deballasted into the EC and GL were from the Atlantic Ocean. Different patterns of bacterial abundances among the deballasting locations were observed in each year. There was no significant difference in bacterial abundance of ballast water samples among locations in 2007. However, the bacterial abundance was higher in ballast water that was deballasted into the EC than in the WC and the GL in 2008. Our results indicate that ocean sources, as proposed by Burkholder et al. (2007), might not be the primary factors for the bacterial propagule pressure distribution among Canadian waters.

### 2.5.7 Cell Volume Comparison between Years

Bacterial cell volumes in the samples collected during 2007 were consistently larger than those collected in 2008 at each deballasting region for each sample type (port, TOE, ICE and NonMOE). Since the cell volume measurements were made by the same person for both years, and the operations were semi-automated, the observed differences were not likely due to observer/analytical variability. Our preservation duration experiment showed

that the cell volume increased with an exponential rate of 5% daily. In our study, the preservation duration of 2007 samples (average 3.2 days) was about 1 day longer than that of 2008 samples (average 2 days). The difference in preservation duration alone could not explain the observed difference in cell volume between the two years. The cell volumes of 2007 samples (Mean ± SD: 0.083 ± 0.021 $\mu m^3$) were about 50% larger than those of 2008 samples (Mean ± SD: 0.055 ± 0.008 $\mu m^3$). The size distribution of bacterial communities is influenced by microzooplankton grazing (Hahn and Hofle 2001). A previous study about bacterial volumes from different natural samples taken along the Mediterranean coast suggested that medium-sized cells with the highest growth rates are subjected to intense grazing (Bernard et al. 2000). Changes in the bacterial size distribution to "inedible" small cells, or less desirable large cells due to microzooplankton grazing have been described both in the field and in the laboratory (Jürgens and Güde 1994; Gasol et al. 1995). Cell volume is an indicator of bacterial activity (Gasol et al. 1995). By directly measuring the size distribution of active (cells that absorbed and reduced the redox dye CTC) and inactive cells in a natural coastal bacterial community, Gasol et al. (1995) found that bacterial activity is a function of cell volume. In their study, the average size of an inactive bacterium was 0.055 $\mu m^3$, while the average size of an active bacterium was 0.12 $\mu m^3$. In this study, the smaller cell size of 2008 samples suggests that bacterial communities might survive in adverse conditions or experience intensive grazing pressure. In contrast, the larger cell size of 2007 samples might indicate higher cell activity due to replete nutrient supply and less grazing pressure.

### 2.5.8 Physiochemical Parameters and Bacterial Variables

Among the measured physiochemical conditions (temperature, salinity, pH, and ballast water age), an inverse relationship was recorded between the bacterial abundance of ICE samples and ballast age. It is premature to unequivocally relate one environmental forcing to the dynamics of bacterial abundance or cell volume of ballast water samples. The lack of consistent relationships between measured environmental parameters and bacterial variables in all three ballast water types indicates there are regulatory factors, other than those which were measured, influencing the bacterial population in ballast water. The dissolved oxygen in ballast water, which was not measured in this study, during voyage might be an influential factor for the dynamics of bacterial community (Tamburri et al. 2002, 2003).

Previous studies documented that species richness and biomass of fish (Wonham et al. 2000), zooplankton (Gollasch et al. 2000), and phytoplankton (Burkholder et al. 2007) decreased as ballast water aged. With the potential augmentation of dissolved organic matter (DOM) in ballast water from planktonic constituents decomposing, the bacterial abundance in ballast water samples did not flourish as expected. In contrast, the bacterial abundances of ICE samples decreased with ballast water age. Similarly, a previous study on bacterial community dynamics during shipping voyage from Israel to the USA also indicated that the bacterial abundance declined with an increase in ballast water age (Drake et al. 2002). Drake et al. (2002) have proposed three probable non-exclusive reasons: (1) the DOM from the phyto- and zooplankton die-off could not be utilized by

bacteria efficiently; (2) bacteria are removed by microzooplankton grazing; or (3) the pulse of DOM from phyto- and zooplankton die-offs is respired by bacteria in the beginning of the voyage, and the remaining small amount of DOM in the holds leads to a steady state of low microbial biomass through the voyage. Besides biological reasons, another possible explanation could be that the bacterial die-off is a response to toxic metal corrosion from the interior ballast tanks.

This study primarily investigated commercial bulk carriers and tankers, which carry the largest volumes of ballast water that are deballasted into Canadian jurisdictions (Harvey et al. 1999). Sampling ports that were chosen are major Canadian ports either in ship traffic or in the ballast water discharged volume among Canadian ports (Harvey et al. 1999). Sampling times spanned from spring to fall, which are the most intense shipping seasons into Canada (Balaban 2001). Therefore, our study supplied an opportunity to understand the roles of commercial shipping activities in redistributing globally bacteria along Canadian waters. With the estimation of 40 million metric tons of ballast water annually deballasted in Canada (Lo et al. 2008), and average bacterial abundance $8.5 \times 10^8$ cells $L^{-1}$ in ballast water, $3.3 \times 10^{19}$ prokaryotic cells are transported into Canadian ports annually.

## 2.6 Conclusion and Future Directions

Here we present results of bacterial abundances and cell volumes for ballast and port water samples from the West and East coasts of Canada and the Great Lakes, and how

these bacterial parameters are related to different ballast water managements, and physiochemical factors. Overall, the consistently lower bacterial abundances in ballast than in port water samples in our study showed that ballast water does not enhance bacterial biomass in ports. However, considering the huge volume of ballast water transported internationally and the high bacterial abundances in ballast water ($10^7$ - $10^9$ cells $L^{-1}$), ballast water may be an important contributor for the ubiquitous distribution of certain phylogenetic groups of bacteria. Among the ballast water types, our results indicate that unexchanged ballast water introduces higher propagule pressure than ballast water exchanged at sea. Higher propagule pressure of bacteria was introduced into the EC than the WC and GL in 2008, but no propagule pressure difference was observed among the three locations in 2007. The larger cell volumes in 2007 than in the 2008 samples suggests higher cell activity or/and lower grazing pressure during 2007. An inverse relationship was recorded between the bacterial abundance of ICE samples and ballast age. The lack of consistent relationships between measured physiochemical and bacterial variables in all three ballast water types indicates there are regulatory factors, other than those which were measured, influencing the bacterial population in ballast water.

Although ballast water introduces large numbers of bacteria into ports, the fate of bacteria after being discharged from ballast tanks, and regional susceptibility to invasion are poorly understood. It is critical to know who is there, and which phylogenetic groups are present in receiving ports relative to those introduced via ballast water (Carlton 1996). Thus, further studies to compare bacterial community composition, species abundance and their physiochemical conditions between ballast and port water samples are necessary

to evaluate the risk of bacterial invasion by ballast water. Furthermore, previous studies show ships can globally distribute pathogens, such as V*ibrio cholerae,* and *Escherichia coli* (Ruiz et al. 2000). It will be important to investigate the existence and abundances of pathogens in ballast water to strengthen insights about the potential transport of pathogenic microbes by ballast water.

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Table 2.1 Sample number according to sampling years (2007, 2008), sampling locations (Great Lakes, West Coast and East Coast of Canada), and sample categories.

| Year | Sampling Location | port | MOE | | NonMOE | Sum |
|---|---|---|---|---|---|---|
| | | | TOE | ICE | | |
| 2007 | West Coast | 3 | 14 | 8 | 6 | 31 |
| | East Coast | 5 | 19 | 4 | 0 | 28 |
| | Great Lakes | 1 | 8 | 3 | 1 | 13 |
| 2008 | West Coast | 10 | 11 | 14 | 16 | 51 |
| | East Coast | 11 | 2 | 19 | 6 | 38 |
| | Great Lakes | 2 | 8 | 0 | 0 | 10 |
| Sum | | 32 | 62 | 38 | 29 | 161 |

**Note:**

Port water samples (port), trans-oceanic exchanged ballast water (TOE), intra-coastal exchanged ballast water (ICE), and without mid-ocean exchange ballast water (NonMOE).

Table 2.2 Bacterial abundances ($10^9$ cells $L^{-1}$) and cell volumes ($\mu m^3$) in three ballast water types, three sampling regions and two years.

| Year | Sampling Location | Ballast Water Types[1] | n | Bacterial Abundance | | | | | Cell Volume | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Min. | Max. | Mean | 95% CI | | Min. | Max. | Mean | 95% CI | |
| | | | | | | | LL | UL | | | | LL | UL |
| 2007 | East Coast | TOE | 19 | 0.09 | 1.75 | 0.68 [a] | 0.49 | 0.88 | 0.051 | 0.119 | 0.074 [a] | 0.065 | 0.083 |
| | | ICE | 4 | 0.71 | 1.08 | 0.82 [a] | 0.55 | 1.10 | 0.058 | 0.090 | 0.073 [a] | 0.051 | 0.096 |
| | | NonMOE | 0 | | | | | | | | | | |
| | Great Lakes | TOE | 8 | 0.45 | 1.03 | 0.82 [a] | 0.63 | 1.01 | 0.062 | 0.163 | 0.100 [a] | 0.073 | 0.128 |
| | | ICE | 3 | 0.62 | 1.02 | 0.79 [a] | 0.27 | 1.30 | 0.069 | 0.119 | 0.093 [a] | 0.030 | 0.155 |
| | | NonMOE | 1 | | | 0.93 [na] | | | | | 0.075 [na] | | |
| | West Coast | TOE | 14 | 0.25 | 1.88 | 0.61 [b] | 0.32 | 0.89 | 0.064 | 0.102 | 0.079 [b] | 0.072 | 0.087 |
| | | ICE | 8 | 0.28 | 1.19 | 0.67 [ab] | 0.46 | 0.88 | 0.069 | 0.120 | 0.097 [a] | 0.084 | 0.110 |
| | | NonMOE | 6 | 0.74 | 2.09 | 1.15 [a] | 0.47 | 1.82 | 0.061 | 0.106 | 0.077 [ab] | 0.053 | 0.101 |
| 2008 | East Coast | TOE | 2 | 0.76 | 1.27 | 1.01 [na] | | | 0.062 | 0.088 | 0.075 [na] | | |
| | | ICE | 19 | 0.66 | 2.60 | 1.16 [a] | 0.96 | 1.36 | 0.048 | 0.061 | 0.054 [a] | 0.052 | 0.056 |
| | | NonMOE | 6 | 0.82 | 1.81 | 1.37 [a] | 0.94 | 1.81 | 0.044 | 0.065 | 0.054 [a] | 0.046 | 0.062 |
| | Great Lakes | TOE | 8 | 0.53 | 0.87 | 0.70 [na] | 0.60 | 0.79 | 0.047 | 0.067 | 0.057 [na] | 0.052 | 0.062 |
| | | ICE | 0 | | | | | | | | | | |
| | | NonMOE | 0 | | | | | | | | | | |
| | West Coast | TOE | 11 | 0.37 | 0.80 | 0.60 [b] | 0.51 | 0.68 | 0.051 | 0.074 | 0.059 [a] | 0.054 | 0.064 |
| | | ICE | 14 | 0.34 | 1.62 | 0.84 [ab] | 0.68 | 1.00 | 0.041 | 0.066 | 0.054 [ab] | 0.050 | 0.059 |
| | | NonMOE | 16 | 0.55 | 1.61 | 0.92 [a] | 0.77 | 1.07 | 0.039 | 0.063 | 0.050 [b] | 0.045 | 0.055 |

***Notes:***

"1": Trans-oceanic exchanged ballast water (TOE), intra-coastal exchanged ballast water (ICE), and without mid-ocean exchange ballast water (Non-MOE).

Analyses of Variances were used to determine overall differences in bacterial abundances and cell volumes among ballast water types at each location and each year. Tukey 95% Simultaneous Confidence Intervals were used to determine the differences among categories.

"a", "b", "na" and "ab": Significant differences among mean values of ballast water types at each locations and each year are indicated by superscript codes: mean with superscript "a" has significantly larger value than that with superscript "b"; "ab" indicate there are no significant difference between means with "a" and "ab", or between means with "b" and "ab"; means with same superscript are not significant different; mean with "na" was not compared because of the limited sample numbers.

Table 2.3 Bacterial abundances and cell volumes of each sample category comparison between 2007 and 2008 at each location.

| Sampling Location | Category [1] | Bacterial Abundance 2007 vs 2008 | | Cell Volume 2007 vs 2008 | |
|---|---|---|---|---|---|
| | | **F value** | **P values** | **F values** | **P values** |
| East Coast | port | $F_{1, 14} = 2.62$ | 0.128 | $F_{1, 14} = 21.04$ | **<0.001** |
| | TOE | - | - | - | - |
| | ICE | $F_{1, 21} = 2.40$ | 0.136 | $F_{1, 21} = 28.63$ | **<0.001** |
| | NonMOE | | - | | - |
| Great Lakes | port | - | - | - | - |
| | TOE | $F_{1, 14} = 1.73$ | 0.209 | $F_{1, 14} = 13.62$ | **0.002** |
| | ICE | - | - | - | - |
| | NonMOE | - | - | - | - |
| West Coast | port | $F_{1, 11} = 0.62$ | 0.448 | $F_{1, 11} = 49.39$ | **<0.001** |
| | TOE | $F_{1, 23} = 0.01$ | 0.905 | $F_{1, 23} = 21.38$ | **<0.001** |
| | ICE | $F_{1, 20} = 1.31$ | 0.266 | $F_{1, 20} = 76.61$ | **<0.001** |
| | NonMOE | $F_{1, 20} = 2.09$ | 0.164 | $F_{1, 20} = 26.18$ | **<0.001** |

**Notes:**

1. Port water samples (port), trans-oceanic exchanged ballast water (TOE), intra-coastal exchanged ballast water (ICE), and without mid-ocean exchange ballast water (Non-MOE).

"ns": no significant difference

"-": no comparison was conducted due to insufficient samples

Table 2.4 Bacterial abundances ($10^9$ cells $L^{-1}$) and cell volumes ($\mu m^3$) in ballast water samples and port water samples at each sampling region each year.

| | | | | Bacterial Abundance | | | | | Cell Volume | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | 95% CI | | | | | 95% CI | |
| Year | Sampling Location | Category | n | Min. | Max. | Mean | LL | UL | Min. | Max. | Mean | LL | UL |
| 2007 | East Coast | Port | 5 | 0.34 | 1.97 | 1.11 [a] | 0.31 | 1.90 | 0.074 | 0.134 | 0.096 [a] | 0.065 | 0.126 |
| | | Ballast | 23 | 0.09 | 1.75 | 0.71 [a] | 0.55 | 0.87 | 0.051 | 0.119 | 0.074 [b] | 0.066 | 0.082 |
| | Great Lakes | Port | 1 | | | 10.06 [na] | 0.00 | 0.00 | | | 0.066 [na] | | |
| | | Ballast | 12 | 0.45 | 1.03 | 0.82 [na] | 0.69 | 0.95 | 0.062 | 0.163 | 0.096 [na] | 0.078 | 0.115 |
| | West Coast | Port | 3 | 1.72 | 2.73 | 2.31 [a] | 1.00 | 3.62 | 0.078 | 0.093 | 0.087 [a] | 0.068 | 0.106 |
| | | Ballast | 28 | 0.25 | 2.09 | 0.74 [b] | 0.56 | 0.93 | 0.061 | 0.120 | 0.084 [a] | 0.078 | 0.090 |
| 2008 | East Coast | Port | 11 | 1.36 | 7.64 | 2.82 [a] | 1.28 | 4.35 | 0.046 | 0.072 | 0.058 [a] | 0.052 | 0.064 |
| | | Ballast | 27 | 0.66 | 2.60 | 1.19 [b] | 1.03 | 1.36 | 0.044 | 0.088 | 0.055 [a] | 0.052 | 0.059 |
| | Great Lakes | Port | 2 | 1.08 | 1.17 | 1.13 [na] | 0.00 | 0.00 | 0.051 | 0.057 | 0.054 [na] | | |
| | | Ballast | 8 | 0.53 | 0.87 | 0.70 [na] | 0.60 | 0.79 | 0.047 | 0.067 | 0.057 [na] | 0.052 | 0.062 |
| | West Coast | Port | 10 | 0.75 | 3.35 | 1.86 [a] | 1.20 | 2.52 | 0.052 | 0.069 | 0.058 [a] | 0.054 | 0.062 |
| | | Ballast | 41 | 0.34 | 1.62 | 0.81 [b] | 0.72 | 0.89 | 0.039 | 0.074 | 0.054 [a] | 0.051 | 0.057 |

**Notes:**

"a", "b"and "na": Significant differences among mean values of ballast water and port water at each locations and each year are indicated by superscript codes: mean with superscript "a" has significantly larger value than that with superscript "b"; means with same superscript "a" are not significant different; mean with "na" was not compared because of the limited sample numbers.

"LL" and "UL": lower limit and upper limit of 95% confidence intervals

Table 2.5 Summary of published studies about bacterial abundance in ballast water

| Sampling | Bacterial Abundance | Influential factors | Notes | References |
|---|---|---|---|---|
| End of voyage: 62 samples from 28 ships along US coastal line | mean ± S.D.: $3.1 \pm 0.5 \times 10^8$ cells $L^{-1}$ | Bacterial abundance was unrelated to vessel type, exchange status, age of water, environmental conditions measured (pH, DO, turbidity, nutrients), or phytoplankton abundance. | Bacterial abundance was significantly lower in ballast tanks with Atlantic than Pacific Ocean source water | Burkholder et al. 2007 |
| End of voyage: vessels arriving to Chesapeake Bay from foreign ports. | mean ± S.D.: $8.3 \pm 1.7 \times 10^8$ cells $L^{-1}$ | | | Ruiz et al. 2000 |
| End of voyage: 25 bulk carriers originating in foreign ports and arriving in Chesapeake Bay | $5.7 \times 10^7$ – $2.0 \times 10^9$ cells $L^{-1}$, except for one sample with abundance $15 \times 10^9$ cells $L^{-1}$ | Bacterial abundance was uncorrelated with temperature, and water age, but negatively correlated with salinity | | Drake et al. 2001 |
| During voyage: vessel from Hadera, Israel to Baltimore USA on a 19 - day voyage. | $9.2 - 22 \times 10^7$ cells $L^{-1}$ | Bacterial abundance decreased by a factor of 2.3 (unexchanged tanks) and 1.6 (exchange tanks) throughout the voyage. There was no difference in bacterial abundance between exchanged tanks and unexchanged tank at the end of voyage | | Drake et al. 2002 |
| End of voyage: ships arrived at Singapore Harbor | $2.35 \times 10^9$ - $5.87 \times 10^{10}$ cells $L^{-1}$ | | Bacterial abundance in ballast water was lower than that in local seawater | Joachimsthal et al. 2004 |
| **Sampling** | **Bacterial Abundance** | **Influential factors** | **Notes** | **References** |
| End of voyage: 69 vessels arriving at lower Chesapeake Bay from foreign and domestic ports | No concentration reported | No bacterial abundance difference between exchanged and unexchanged tanks | Bacteria discharged from vessels and surviving in the Port is $3.9 \times 10^{18}$ cells per year. | Drake et al. 2007 |

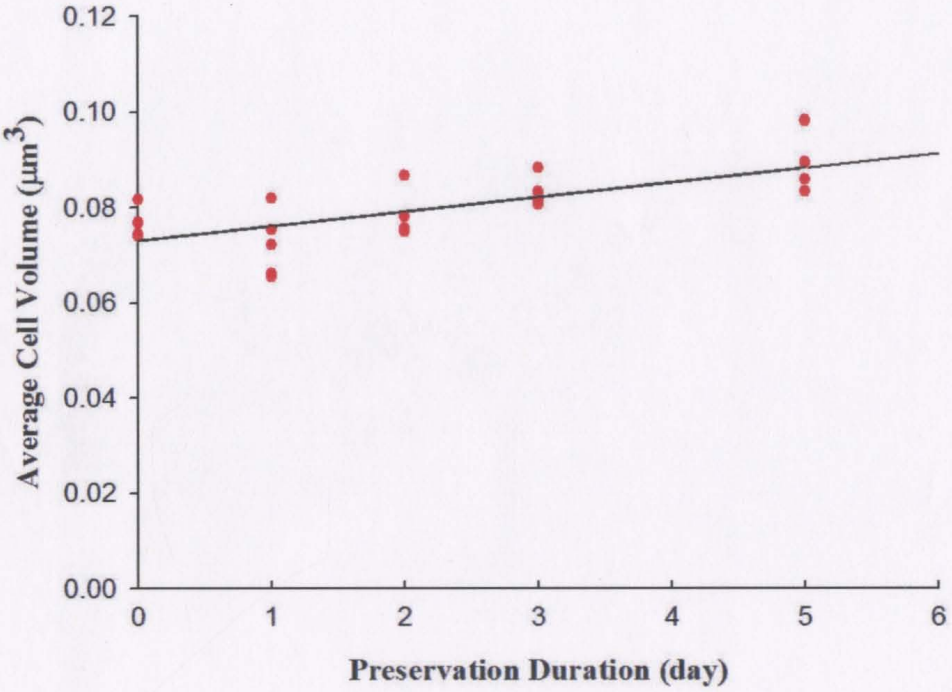


Figure 2.1 Relationship between cell volumes (3.7% formaldehyde preserved samples) and preservation duration (number of days between fixative addition and sample filtration) (Cell Volume = 0.0731 + 0.0030 Duration, $r^2 = 0.52$, $P < 0.001$). Cell volumes of samples preserved for 2 hours (0 day) represented the *in situ* cell volumes.

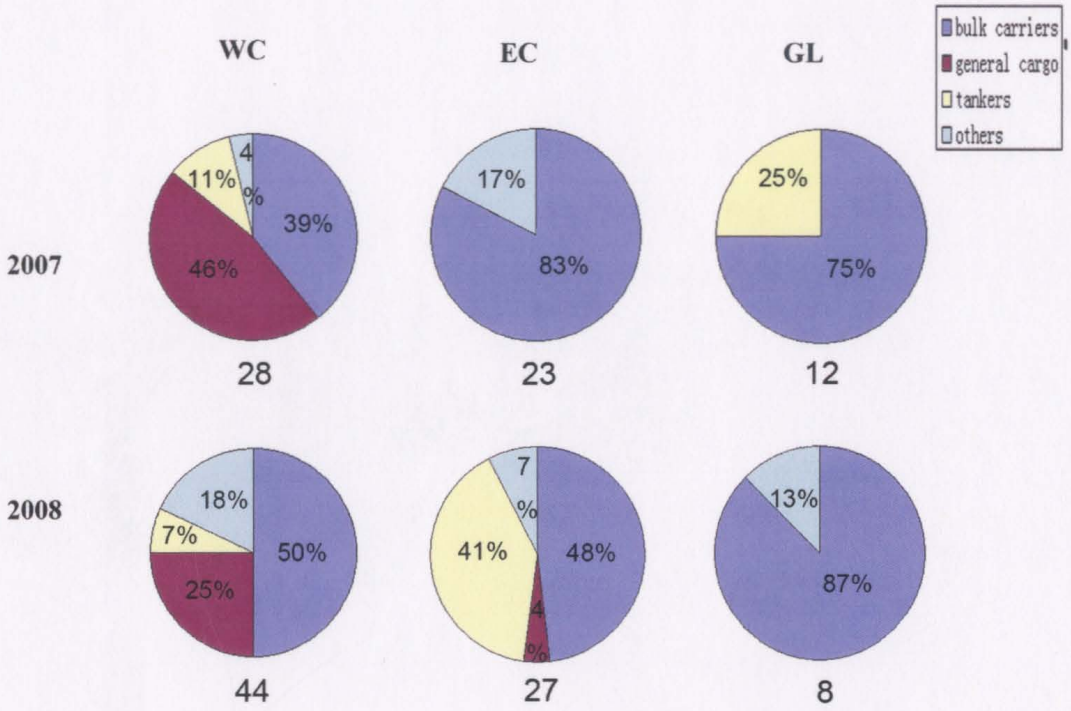


Figure 2.2 The number of ballast water samples (below) and the percentages by vessel types (bulk carriers, general cargo, tankers and others) at the West Coast of Canada (WC), East Coast of Canada (EC), and Great Lakes (GL) sites during 2007 (upper panel) and 2008 (lower panel) sampling seasons. Data on vessel types was collected from ballast water management forms.

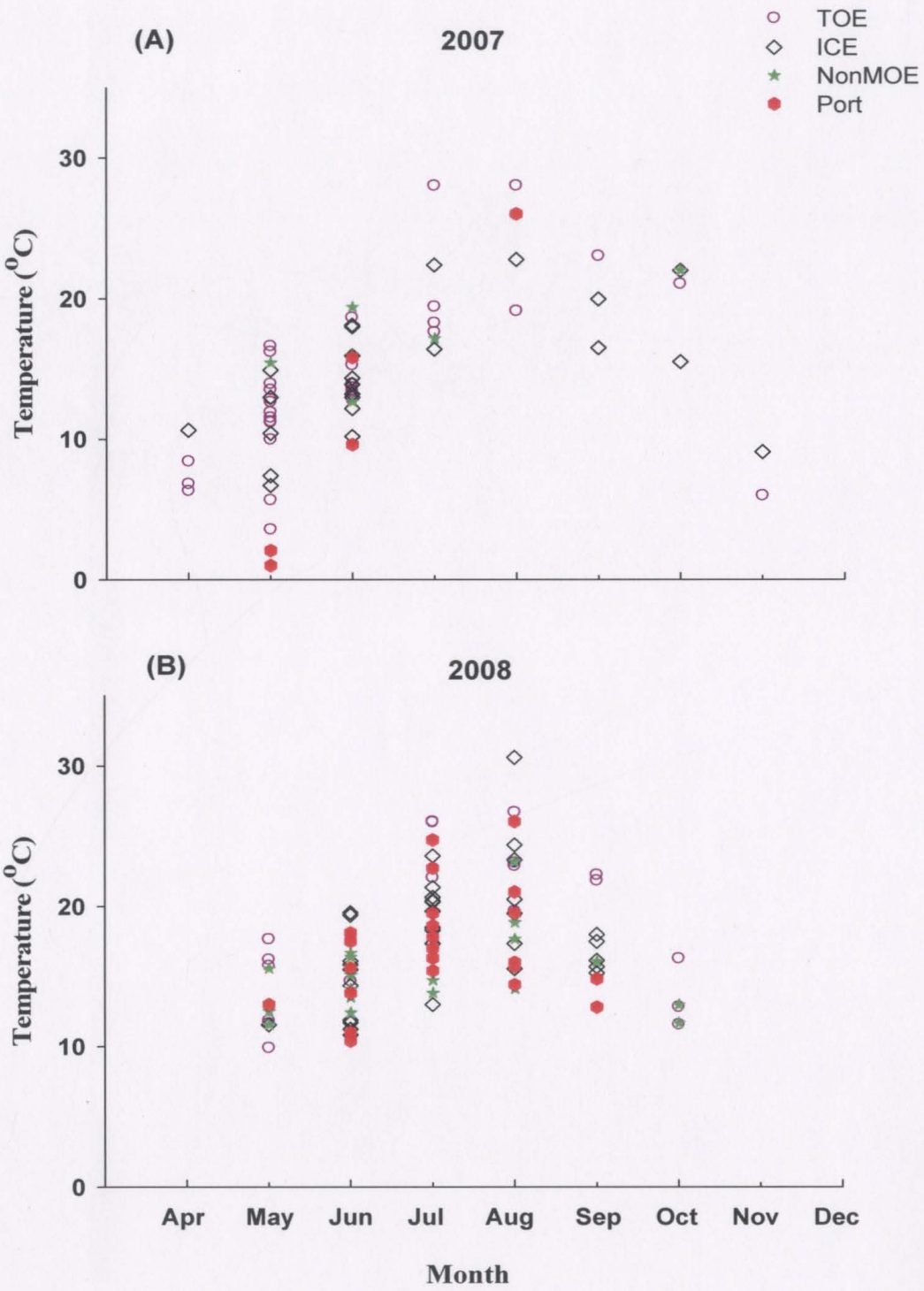
(A)
2007
TOE
ICE
NonMOE
Port
Temperature ($^0$C)
30
20
10
0
(B)
2008
Temperature ($^0$C)
30
20
10
0
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Month

Figure 2.3 Temperature of trans-oceanic exchanged (TOE, open circle) ballast water, intra-coastal exchanged (ICE, open diamond) ballast water, without mid-ocean exchange (NonMOE, closed star) ballast water, and port water samples (port, closed hexagon) monthly distribution in 2007 (upper panel) and 2008 (lower panel) sampling seasons.

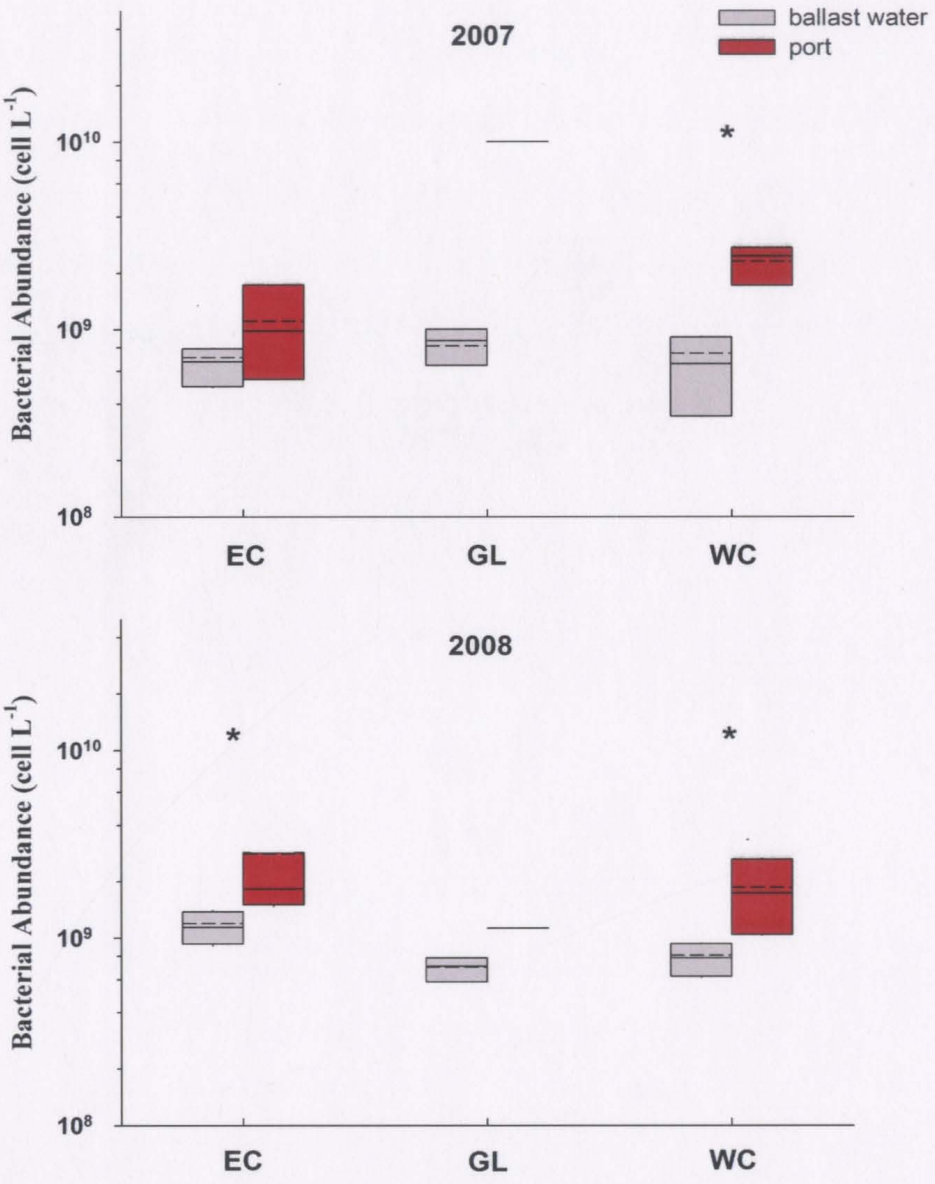
2007
ballast water
port
Bacterial Abundance (cell L-1)
10^10
10^9
10^8
*
*
EC
GL
WC
2008
Bacterial Abundance (cell L-1)
10^10
10^9
10^8
*
*
EC
GL
WC

Figure 2.4 Bacterial abundance of ballast (grey) and port (red) water samples for the West Coast (WC), East Coast (EC), and Great Lakes (GL) in 2007 (upper panel) and 2008 (lower panel). The lower and upper boundaries of each box are the 25th and 75th percentile, respectively. The dashed and solid lines within the boxes are the mean and the median, respectively. "*" indicates that the bacterial abundance was significantly higher in port than in ballast water samples (ANOVA, $p < 0.05$).

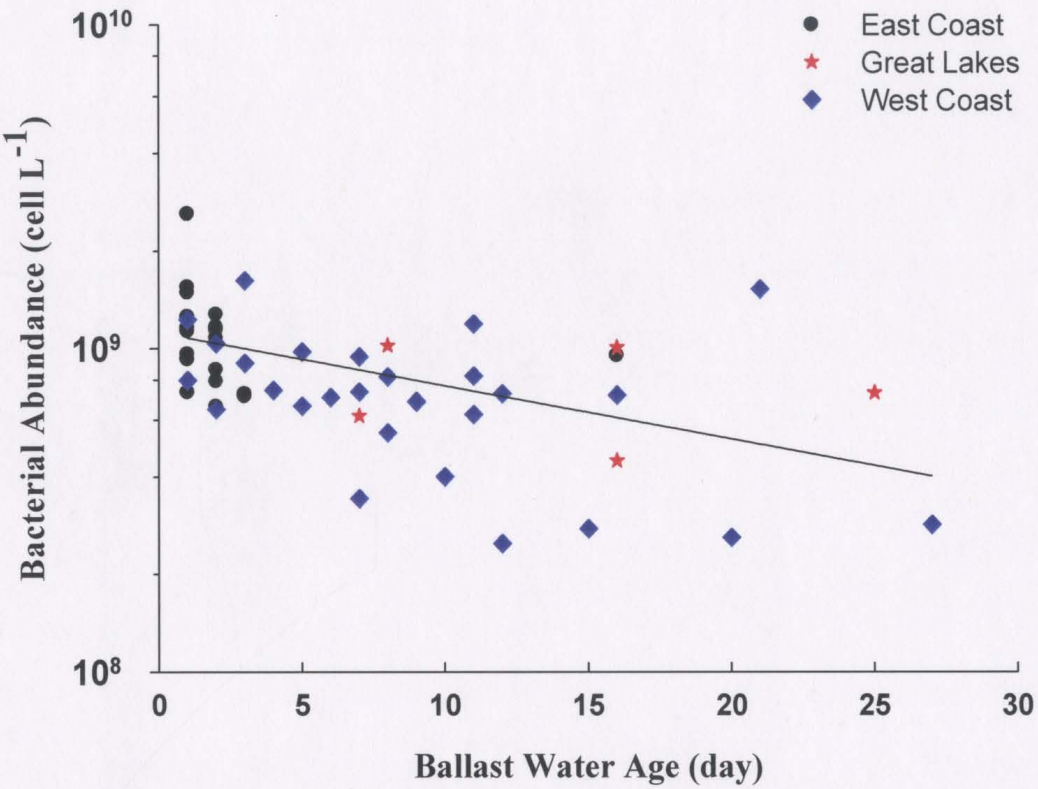


Figure 2.5 Relationship between the bacterial abundance of intra-coastal exchanged (ICE) ballast water samples and ballast water age (number of days between the date of exchange and sampling date) sampled from the Great Lakes (red star), West (blue diamond), and East Coast (black circle) of Canada (Bacterial abundance = $(1.12 \times 10^9)\ e^{-0.04 \times \text{Age (day)}}$, $r^2 = 0.20$, $P < 0.001$) during 2007 and 2008.

# Chapter 3 Bacterioplankton Communities Distributed Globally by Ballast Water

## 3.1 Abstract

Ballast water is one of the primary vectors for the global transport of vegetative and resting stages of pathogenic and non-pathogenic aquatic microorganisms. As part of the Canadian Aquatic Invasive Species Network, the community structures of heterotrophic prokaryotes (hereafter bacteria) in ballast and receiving port waters were characterized along the West and East coasts of Canada and from the Great Lakes. Microbial community structure was characterized using fluorescence *in situ* hybridization. The % *Bacteria* in port and ballast water ranged from 34 to 78%, and 20 to 78%, respectively, of the total bacteria detected. The % *Archaea* was low, and were only found in ballast and port water from the West Coast. *Cytophaga-Flavobacteria* and Alpha-Proteobacteria accounted for more than half of the *Bacteria* in both port and ballast water samples. *Vibrio spp.* was only detected in nine out of 59 ballast water samples. *Escherichia coli* was below detection using FISH, but its presence at low abundance could not be excluded. Negative relationships between ballast water age and the % *Bacteria*, % Alpha-Proteobacteria in ballast water samples implies that the proportion of metabolically active *Bacteria* and Alpha-Proteobacteria in ballast water may have declined with ballast water age. The three ballast water types defined by the Canada ballast water management regulations did not differ in the bacterial community structures introduced at each deballasting region. However, the distinct bacterial community structures between ballast

and those in receiving port water samples have been observed, which implies that there is potentially an environmental risk from ballast water distributed into Canadian harbors.

## 3.2 Introduction

Over 80% of the world's cargo is mobilized internationally by sea-going ships. Besides cargo, over 12 billion metric tons of ballast water is moved across coastal and oceanic domains annually (Anil et al. 2002). Ballast water is one of the primary vectors for the global transport of vegetative and resting stages of aquatic microorganisms (Carlton and Geller 1993; Ruiz et al. 2000), as well as bacterial pathogens, such as *Vibrio cholerae O1* and *O139* (Ruiz et al. 2000). Heterotrophic prokaryotes (hereafter bacteria), including both *Bacteria* and *Archaea*, are abundant and ubiquitous in the World Oceans (Sherr and Sherr 2000), and their abundances have been reported as $10^7$ to $10^{10}$ cells $L^{-1}$ in aquatic environment (Whitman et al. 1998) and $10^7$ to $10^9$ cells $L^{-1}$ in ballast water (Ruiz et al. 2000).

Bacteria that are redistributed in ballast water around the world could impact both the bacterial communities and, consequently, the ecological functions of receiving waters. Bacteria have a central role in mediating biogeochemical processes of the ocean, including the cycling of organic carbon, nitrogen, sulfur, phosphorus and other organic and inorganic elements (Fenchel et al. 1998; Newman and Banfield 2002). Until recently, studies of the role of bacteria in mediating water column biogeochemical processes have viewed bacteria as a single group, neglecting the phylogenetic diversity of bacteria

communities (Kirchman 2002). However, specific bacterial phylotypes differ in their contribution to the marine biogeochemical processes (Fuhrman 2009). For example, the *Cytophaga-Flavobacteria* cluster comprises the largest fraction of cells assimilating chitin and protein, but the smallest fraction consuming amino acids. In contrast, the assimilation of amino acids is dominated by the Alpha-Proteobacteria, for which protein consumption was lowest (Cottrell and Kirchman 2000). The first step to understanding the function of bacterial communities is to study the abundance and activity of different phylogenetic groups within bacterial assemblages (Cottrell and Kirchman 2000; Malmstrom et al. 2005). A study comparing the bacterial phylogenetic structure of ballast water and receiving port water will be valuable in understanding the potential ecological impacts of ballast water introduced bacteria to receiving regions.

To attenuate the impacts of ballast water-mediated organisms, the International Maritime Organization (IMO) established mid-ocean ballast water exchange (MOE) guidelines in 1991. Under these guidelines, Canada initiated mandatory mid-ocean exchange (MOE) for ocean-going ships entering all Canadian ports as of June 8$^{th}$, 2006 (Transport Canada 2006). However, voyages from nearby US ports travelling to Canada do not require MOE because nearby ports would have similar community compositions. Commercial ships, which apply mid-ocean exchange management for ballast water, are divided into transoceanic navigation and intra-coastal navigation. Transoceanic ships are required to exchange their ballast water greater than 200 nautical miles from shore where the water depth is at least 2000 m. In contrast, intra-coastal ships are required to exchange ballast water at least 50 nautical miles from shore and at water depth of at least 500 m (Transport

Canada 2006). Therefore, ballast water from ships arriving from international destinations falls into three categories: trans-oceanic exchanged (TOE); intra-coastal exchanged (ICE); without mid-ocean exchange (NonMOE).

As part of the Canadian Aquatic Invasive Species Network (CAISN), during 2007, a study was carried out to examine the bacterial community structure in ballast water (TOE, ICE, and NonMOE) and receiving port water (i.e. Great Lakes, West Coast and East Coast of Canada) where ships discharge their ballast water. The objectives of this study are to: (1) evaluate the effects of different ballast water exchange protocols (ICE, TOE and NonMOE) on bacterial communities in ballast waters; (2) assess the differences in bacterial community structure between ballast water and receiving ports in each region; (3) assess if there are differences in ballast water bacteria discharged into three sampling regions, and (4) assess the relationships between bacterial community composition and measured environmental factors (i.e. temperature, salinity, pH, and ballast water age).

## 3.3 Materials and Methods

### 3.3.1 The Effects of Preservation on Community Structure

The bacterial community structure was characterized by fluorescence *in situ* hybridization (FISH). This technique, which applies fluorescently-labeled rRNA-targeted oligonucleotide probes designed to hybridize to group-specific gene sequences, is widely used to characterize the phylogenetic diversity of unicellular organisms at target taxonomic levels (Glöckner et al. 1996). Samples that are preserved for FISH should

retain all cellular rRNA content, protect cell integrity and morphology, and allow probe penetration during hybridization (Lam and Cowen 2004). When used as a fixative, formaldehyde forms DNA-protein cross-links within the cells (Bullock 1984). Therefore, the effectiveness of target cell detection could be impaired in over-fixed samples, due to the blockage of probe access to the targeted rRNA molecules. The standard protocol for FISH is to filter a sample after the sample has been fixed for 1-6 h with 0.2 μm prefiltered 3.7% formaldehyde (Glöckner et al. 1996). However, samples shipped from the sampling sites to the laboratory in St. John's, NL, where samples are processed and filtered, can take up to five days.

To determine if samples that are preserved for up to five days can be reliably analyzed for bacterial community structure using FISH, a 10-L seawater sample was collected from Logy Bay, Newfoundland, Canada (47°37'30.32"N, 52°39'48.36"W), on March 13th, 2007. Seawater was preserved in formaldehyde (final concentration 3.7%) in five 1-L bottles and stored at 4°C. A sub-sample (100 ml) from each bottle was analyzed at 2 h and 5 d to compare bacterial community structure. Bacterial community structure at 2 h represents the *in situ* condition. Each seawater sample was filtered onto a white 0.2-μm polycarbonate membrane filter (Millipore GTTP04700, Billerica) that was placed over a cellulose prefilter (Millipore AP1504700, Billerica). Each polycarbonate filter was washed with 50 ml of saline phosphate buffer (sterile and prefiltered), and air-dried over absorbent paper in individual sterile Petri dish. Filters were stored in parafilm-sealed Petri dishes at -20°C until the analysis of prolonged fixation (five days) effects on bacterial community structure with FISH.

### 3.3.2 Ballast Water and Port Water Sampling

Sampling was carried out from March 2007 to November 2007 from three sampling regions of Canada (EC-East Coast; GL-Great Lakes; WC-West Coast) that actively engage in international shipping (Lo et al. 2007). These sites are the West Coast (Vancouver, British Columbia) and the East Coast of Canada (Baie-Comeau, Sept-Iles, Port-Cartier, all on the lower north shore of the St Lawrence estuary, Quebec), and the Great Lakes (Toledo, Ohio, USA; Milwaukee, Wisconsin, USA; Detroit, Michigan, USA; and Sarnia, Ontario, Canada). Commercial vessels that arrived at those ports and had ballast tanks with TOE, ICE, and NonMOE, were sampled. Port water samples were periodically collected. The sampling goals were 10 samples in each of the TOE, ICE and NonMOE categories from the West Coast of Canada and East Coast Canada, each region, and 10 MOE (TOE or ICE) samples from the Great Lakes.

For each ballast tank, samples were collected, through a deck hatch, by a Niskin bottle lowered to four depths in the tank (surface, mid surface, mid bottom, and bottom). Equal volumes of ballast water from each of the sampling depths were combined together from the same ballast tank. Associated environmental data (temperature, salinity, and pH) was also recorded from each sampling depth using a handheld YSI Model 85 meter equipped with a 15 m cable (YSI Incorporated, Yellow Springs, OH, USA). A sub-sample (500ml) of the combined ballast water from one tank was preserved with formaldehyde (final concentration 3.7%) and shipped on ice to the Ocean Sciences Centre (St. John's, NL) for

analyses within five days. Along with the collected ballast water samples, the ballast water management forms with information on vessel type, ballast water source, exchange location, ballast water deballast port, onboard ballast water volume, exchanged ballast water volume and deballast water volume of sampled vessels data were provided.

### 3.3.3 Sample Filtration

After a sample was received, 5 ml would be filtered onto a 25 mm diameter, 0.2 μm black polycarbonate filter (GE Osmonics Labstore, Minnetonka), stained with acridine orange (AO) (final concentration $1.872\times10^{-5}$g $L^{-1}$) for image analysis to determine the % rod-shaped cells (Hobbie et al. 1977; Kirchman et al. 1982). Another 100ml of the sample was filtered on a polycarbonate membrane filter for FISH analysis, as described above.

### 3.3.4 Image Analysis for Percent Rod-Shaped Cells

The slides for characterizing cell size and morphometrics were observed using an Olympus BH2-RFC epifluorescence microscope, equipped with a 100 x 1.30 oil objective (1250 x total magnification), a 100W mercury lamp and appropriate filter sets (502 nm for excitation, 526 nm for emission). Cell dimensions were determined using an Image-Pro Plus V6.2 Image Analysis System (Media Cybernetics, Inc., Bethesda). The system is configured to capture and store digital images, which are then used for subsequent measurement of the size distribution of cells. The epifluorescence microscope was equipped with a CCD camera linked to a desktop computer. The image analysis system used was digitally calibrated using a stage micrometer and initial calibration

software. Images of AO-stained cells that fluoresce on the slide were captured by the camera and the image (.tif) diverted to a Dell PC. Cell length and cell width as measured, and the images were individually examined, and the cell dimensions were automatically recorded. Manual determination of the fluorescence intensity threshold was essential in determination of cell edge locations. Detritus particles or cells (i.e. clumped or aggregated) were screened out from the analysis either through the direct removal from the working image, or by constraints assigned to acceptable diameters. Slides for individual experiments were made at the same time so that bacterial slide quality and cell sizes within an experiment were comparable. The output measured variables for each sample were downloaded to an Excel spreadsheet. Cells with an aspect ratio <1.5 were regarded as spheres whereas cells with an aspect ratio >1.5 were counted as rod-shaped cells.

### 3.3.5 Oligonucleotide Probes

In this study, fluorescently-labeled rRNA-targeted oligonucleotide probes (Integrated DNA Technologies, Coralville, USA) were used for the phylogenetic analysis of heterotrophic prokaryotes from the domain *Bacteria* (EUB338) and *Archaea* (ARCH915); the intermediate taxonomic levels of *Bacteria*: Alpha-Proteobacteria (ALF968), *Cytophaga-Flavobacteria* (CF319a) cluster; as well as the potential pathogens: *Vibrio spp.* (GV) and *Escherichia coli.* (Eco16S07C). The 'nonsense' probe (NON338), which is complementary to the EUB338 probe, was used as a negative control for the detection of non-specific binding and background fluorescence (Glöckner et al. 1996). The targeted bacterial groups and gene sequences for the oligonucleotide probes employed in this

study are shown in Table 3.1. All oligonucleotide probes were labeled at the 5' end position with the sulfoindocyanine dye, indocarbocyanine (Cy3). *Bacteria* (EUB338), Alpha-Proteobacteria (ALF968) and 'nonsense' (NON338) probes were applied for the study of the effects of prolonged preservation on community structure. The hybridization efficiency of probes GV and Eco16S07C were tested on pure cultures of *Vibrio parahaemolyticus* and *E. coli* JM109 *lambda-pir*, respectively (obtained from the Department of Biology of Memorial University of Newfoundland). The presence of *E. coli* was assessed in 11 samples selected to represent the highest bacterial abundance in each sample type at each deballasting location.

### 3.3.6 Fluorescence *In Situ* Hybridization

A modification of the protocol of Glöckner et al. (1996) was used. Each filter was cut into eight triangular sections, and each section was placed onto a microscope slide. To ensure that the side containing the bacteria was facing upwards, a slit was cut in the right edge of each filter section. The filter sections were hybridized with 50ng of oligonucleotide probe diluted in 25 µl of hybridization solution (0.9 M NaCl, 20 mM pH 8 Tris-HCl, formamide concentration varying for each probe (Table 3.1), and 0.01% sodium dodecyl sulphate). Each slide was placed in a sealed hybridization chamber containing a piece of absorbent paper and 1 ml of hybridization buffer (to create a humid atmosphere), and incubated in the dark for two hours at 46°C. Following incubation, filter sections were placed into individual 50ml sterile glass bottle of pre-warmed (46°C) washing buffer, containing 20 mM Tris-HCl, 5 mM EDTA, 0.01% SDS, and NaCl concentration varying for each probe

(Table 3.1), and incubated in the dark for 15 min at 46°C. Each filter section was dried over absorbent paper at room temperature, placed on a glass slide, and counter-stained with 50 µl of 1 µg $ml^{-1}$ DAPI (4',6-diamidino-2-phenylindole) for 1 minute (on ice). After staining, each filter section was washed with 1 ml of filtered Milli-Q water, dried over absorbent paper and mounted on a glass slide in glycerol medium (Citifluor Ltd; London, UK). The slides were then stored at -20°C (for a maximum of 48 hours) before microscopic analysis.

### 3.3.7 Epifluorescence Microscopy for Community Structure

The slides with the hybridized filter sections were observed using the same epifluorescence microscope described above but equipped with the filter sets for Cy3 (41007-HQ) and DAPI (UG-1). All probes were fluorescently-labeled with the sulfoindocyanine dye Cy3, which absorbs at 552 nm (green light) and emits at 565 nm (orange light). As Cy3 fluorescence fades much more rapidly than DAPI fluorescence (Pernthaler et al. 2001), direct counts of hybridized (group specific) cells were completed first, followed by DAPI counts (under UV light), for total bacteria, in the same field of view. At least 500 hybridized cells were enumerated for each of the filters, or at low abundances of hybridized cells, at least 1,000 DAPI stained cells were counted (Pernthaler et al. 1998). All probe-specific cell counts were presented as the percentage of cells visualized by DAPI. The mean of each sample was calculated from the percentage values of 10 to 20 randomly chosen fields on each filter section.

### 3.3.8 Determination of the Proportion of DAPI Stained Cells Detected as Each Phylogenetic Group

The proportion of DAPI stained cells detected as each target phylogenetic group was determined by dividing the number of hybridized cells for the target phylogenetic group by the total number of DAPI stained cells in each field of view: % of specific phylogenetic group = Probe positive/ DAPI positive. All values were corrected for non-specific binding and background fluorescence by subtracting counts obtained with the Non338 probe. If the adjusted value was less than zero, it was record as zero.

### 3.3.9 Statistical Analyses

Randomized-block ANOVA was used to investigate the effect of the duration of preservation on FISH (Kirk 2003). The response variables were the % *Bacteria* and % Alpha-Proteobacteria, the random blocking explanatory variable was five replicate bottles, and the fixed explanatory variable was fixation duration (2 h or 5d). Multivariate Analyses of Variance (ANOVA) were applied first to analyze the relationships of measured bacterial parameters among different sample types and sampling locations; however, the statistics showed that the interaction terms were significant. Therefore, one-way ANOVA were carried out to compare the measured bacterial parameters (the % rod-shaped cells, the % *Bacteria*, % Alpha-Proteobacteria, and % *Cytophaga–Flavobacteria*) among the three ballast water types, and between ballast and port waters at each deballasting location, and among three deballasting locations. If a statistically significant result was found in an omnibus F-test for a one-way ANOVA,

post-hoc analyses using the Tukey test were conducted. There was no NonMOE samples collected from the EC, and only one port and one NonMOE samples were collected from the GL, which prevented their use in the above statistical comparisons. Multiple regression analyses were carried out to determine the relationships between measured bacterial parameters and environmental variables (i.e. temperature, pH, salinity, ballast water age).

All statistical analyses were conducted using Minitab Release 14 (Minitab Inc., State College). For each analysis, the residuals were examined and met the assumptions of linearity, normality, independence, and homogeneity (Seber and Lee 2003). Therefore, the proportion (percentage) data were not necesssary to transform to arcsin. The significance judge criterion for statistics in this study is $\alpha$=0.05.

## 3.4 Results

### 3.4.1 Effects of Preservation Duration on Community Structure

The preservation experiment was carried out to test the stability of preserved samples for FISH analysis. *Bacteria* and Alpha-Proteobacteria were tested because they are both abundant in marine environment, and also they represent different phylogenetic levels of bacterial community structure. There was no significant difference in either % *Bacteria* or % Alpha-Proteobacteria between the 2 h (i.e., the *in situ* condition) and 5 d preservation duration (% *Bacteria*: $F_{1,4} = 0.72$, $P = 0.443$; % Alpha-Proteobacteria: $F_{1,4} = 0.33$, $P = 0.594$). The % *Bacteria* ranged from 59 to 64% at 2 hours, and from 59 to 61% at 5 days.

The % Alpha-Proteobacteria ranged from 24 to 29% detected at 2 hours, and from 25 to 29% at 5 days.

### 3.4.2 Vessels Sampled and Ballast Water Management

A total of 59 ballast water samples and 10 port water samples were collected. The ballast water samples were primarily from commercial bulk carriers, general cargo, and tankers, with a few container vessels, roll on/roll off and multi-purpose ships (Figure 3.1). The majority of ballast water samples that were collected from the West Coast (85%) of Canada were from bulkers and general cargo, while the majority of ballast water samples from Great Lakes (100%) and the East Coast of Canada (83%) were from bulkers and tankers (Figure 3.1).

### 3.4.3 Percent Rod-Shaped Cells

The % rod-shaped cells among sample types (port, TOE, ICE, and NonMOE) are reported in Table 3.2. ANOVA results show that there was a significant difference in the % rod-shaped cells among port, TOE, and ICE at the EC ($F_{2, 25} = 5.50$, $P = 0.010$, Table 3.2). Post-hoc comparisons using the Tukey test indicated that the % rod-shaped cells was significant higher in port than TOE samples, but there was no significant difference between port and ICE, or between TOE and ICE samples at the EC. There was no significant difference in % rod-shaped cells among ballast water categories at the WC ($F_{3, 27} = 0.42$, $P = 0.742$, Table 3.2); nor between TOE and ICE samples at the GL ($F_{1, 9} = 0.87$, $P = 0.374$, Table 3.2). Too few port and NonMOE samples were collected from the GL,

which prevented their use in the statistical comparison. Taken together, there was no significant difference in % rod-shaped cells among ballast water types in each region.

The % rod-shaped cells between ballast and port water samples at each sampling region are shown in Figure 3.2. ANOVA results showed that the % rod-shaped cells was significantly higher in port than ballast water samples that were deballasted into the EC ($F_{1, 26} = 11.30$, $P = 0.003$, Table 3.2), but there was no difference at the WC ($F_{1, 29} = 0.12$, $P = 0.726$, Table 3.2). Since only one port sample was collected from the GL, the statistical test for differences between port and ballast water sample at the GL was not conducted.

There was no significant difference in the % rod-shaped cells of port samples between EC and WC ($F_{1, 6} = 4.36$, $P = 0.082$, Table 3.2), or in the % rod-shaped cells of the TOE ($F_{2, 38} = 0.26$, $P = 0.776$, Table 3.2) or ICE ($F_{2, 13} = 0.46$, $P = 0.639$, Table 3.2) samples among the three deballasting locations. Due to the low number of NonMOE samples, the statistical test for differences in the % rod-shaped cells in NonMOE samples among locations was not carried out (Table 3.2).

### 3.4.4 Microbial Community Composition Determined by FISH Detection with the Domain Probes: *Bacteria* and *Archaea*

The results of the % *Bacteria* (EUB338) for each ballast water type and each sampling region are shown in Table 3.3. There was no significant difference in the % *Bacteria*

among TOE, ICE, and NonMOE samples at the WC ($F_{2, 22} = 1.28$, $P = 0.299$, Table 3.3), or between TOE and ICE samples at the EC ($F_{1, 20} = 3.71$, $P = 0.069$, Table 3.3) and GL ($F_{1, 9} = 0.11$, $P = 0.748$, Table 3.3). The number of NonMOE samples collected at the EC and GL ports was low, and no statistical analysis was carried out.

The % *Bacteria* was compared between ballast and port water samples. The % *Bacteria* in port water samples ranged from 34 to 78%, with a mean of 66 % (95% CI, 57 to 74%, n=10), and 20 to 78% in ballast water samples, with a mean of 47 % (95% CI, 43 to 50%, n=59). The mean % *Bacteria* were significantly higher in port than ballast water samples at the EC and WC (EC: $F_{1, 25} = 12.07$, $P = 0.002$; WC: $F_{1, 27} = 11.07$, $P = 0.003$, Figure 3.3). Since only one port sample was collected from the GL, statistical analysis of the differences between port and ballast water samples at the GL site was not carried out.

Since there was no significant difference in % *Bacteria* among ballast water types at each of the locations (Table 3.3) and the interactive term between ballast water types and locations was not significant ($F_{3, 51} = 0.745$, $P = 0.530$), the data from all three ballast water types at each location were grouped together as combined ballast water samples. There was no significant difference in % *Bacteria* of combined ballast water samples among deballasting sites ($F_{2, 56} = 0.57$, $P = 0.570$, Table 3.3), or for port water samples between the EC and WC ($F_{1, 7} = 0.03$, $P = 0.871$, Table 3.3).

The % *Archaea* (ARCH915) was low (< 2%) for both ballast water and port water samples in the EC and GL (Table 3.3, Figure 3.3). The % *Archaea* in ballast water

samples deballasted into the WC ranged from 0 to 6%, with the mean of 1.6%; and the % *Archaea* of port samples from the WC ranged from 2 to 12%, with the mean of 6% (Table 3.3, Figure 3.3). The % *Archaea* in ballast water deballasted into the WC is significantly lower than in port water samples ($F_{1, 27} = 10.79$, $P = 0.003$).

## Detection with Group-Specific Probes: Alpha-Proteobacteria and *Cytophaga-Flavobacteria*

The results for the % Alpha-Proteobacteria (ALF968), and % *Cytophaga-Flavobacteria* (CF319a) for each sample category and each sampling region are shown in Table 3.4. There were no NonMOE samples collected from the EC. At the EC site, there was no significant difference in the % Alpha-Proteobacteria among port, TOE and ICE samples ($F_{2, 24} = 3.34$, $P = 0.053$, Table 3.4). ANOVA results showed that there was significant difference in the % *Cytophaga-Flavobacteria* among port, TOE, ICE at the EC ($F_{2, 24} = 8.57$, $P = 0.002$, Table 3.4). Post-hoc comparisons using the Tukey test showed that the % *Cytophaga-Flavobacteria* was significantly higher in port than TOE and ICE samples, but there was no significant difference between TOE and ICE samples at the EC (Table 3.4). There was no significant difference in the % Alpha-Proteobacteria, or % *Cytophaga–Flavobacteria* among sample types at the WC (% Alpha-Proteobacteria: $F_{1, 9} = 0.75$, $P = 0.410$; % *Cytophaga–Flavobacteria*: $F_{1, 9} = 0.67$, $P = 0.435$, Table 3.4); nor between TOE and ICE samples at the GL (% Alpha-Proteobacteria: $F_{3, 25} = 2.76$, $P = 0.063$; % *Cytophaga–Flavobacteria*: $F_{3, 25} = 1.61$, $P = 0.212$, Table 3.4). Too few port and NonMOE samples were collected from the GL, so port and NonMOE samples were not

included in the statistical comparison. Taken together, the % *Cytophaga–Flavobacteria* was significantly higher in port than ballast water samples, but there was no significant difference between ballast water types at the EC site. There was no significant difference in the % *Cytophaga–Flavobacteria* among the sample type comparisons at the WC or GL. There was no significant difference in the % Alpha-Proteobacteria among sample types in each of the three locations.

Since there was no significant difference in % Alpha-Proteobacteria and % *Cytophaga-Flavobacteria* among the ballast water types at each of the locations (Table 3.4) and the interactive term between ballast water types and locations was not significant (% Alpha-Proteobacteria: $F_{3, 51} = 2.245$, $P = 0.094$; % *Cytophaga-Flavobacteria*: $F_{3, 51} = 0.369$, $P = 0.776$), the data from all three ballast water types at each location were combined and compared among regions. ANOVA results showed that there was a significant difference in the % Alpha-Proteobacteria among the three regions ($F_{2, 56} = 4.86$, $P = 0.011$). Post-hoc comparisons using the Tukey test indicated that the % Alpha-Proteobacteria was lower at the GL than the EC and WC, but there was no difference between the EC and WC (Table 3.4). There was no significant difference in % *Cytophaga-Flavobacteria* of the combined ballast water samples among the three deballasting locations ($F_{2, 56} = 0.88$, $P = 0.422$, Table 3.4). ANOVA results showed there was no significant difference in the % Alpha-Proteobacteria or % *Cytophaga-Flavobacteria* of port samples between the EC and WC (% Alpha-Proteobacteria: $F_{1, 7} = 1.17$, $P = 0.315$; % *Cytophaga-Flavobacteria*: $F_{1, 7} = 4.21$, $P = 0.079$).

### Detection of *Vibrio spp.* and *E. coli*

The hybridization efficiencies of the GV and Eco16S07C probes were 100% on strain cultures *Vibrio parahaemolyticus* and *E. coli* JM109 *lambda-pir*, respectively, confirming the suitability of these probes. *Vibrio spp.* were detected in approximately 15% ballast water samples (nine out of 59 samples), with % *Vibrio spp.* ranging from 2 to 17 %. *E. coli* was not detected in the eleven samples tested.

## 3.4.5 Relationships between Microbial Community Structure and Physiochemical Factors

The relationships between bacterial community structure in ballast water samples and physiochemical factors (salinity, pH, temperature, and ballast water age) were examined. Since there was no significant difference in % *Bacteria*, % Alpha-Proteobacteria or % *Cytophaga-Flavobacteria* among the ballast water types at each of the locations (Table 3.3 and Table 3.4), the data from all ballast water types at each location were grouped as combined ballast water samples. The only statistically significant relationships discerned were between the % *Bacteria*, % Alpha-Proteobacteria and ballast water age, and between % *Cytophaga-Flavobacteria* and temperature. Both the % *Bacteria* and the % Alpha-Proteobacteria in ballast water samples decreased with an increase in ballast water age (% *Bacteria*: $P = 0.014$, $r^2=0.10$, $n = 59$, Figure 3.4; % Alpha-Proteobacteria: $P < 0.001$, $r^2=0.24$, $n = 59$, Figure 3.5). The % *Cytophaga-Flavobacteria* in ballast water

samples decreased with the increase of ballast water temperature ($P = 0.015$, $r^2=0.15$, $n = 59$, Figure 3.6).

## 3.5 Discussion

### 3.5.1 Preservation Duration Effects on Community Structure

It took one to five days for samples to be delivered from the sampling sites to the laboratory in St.John's, NL, where they were filtered and processed. Therefore, the effect of short-term sample preservation (5 days) in suspension on the hybridization efficiency and reproducibility was assessed. One possible effect of prolonged preservation could be the blockage, due to the cross-linking structure formed during the preservation, of the probe access to targeted rRNA. However, there was no significant difference in the % *Bacteria* or % Alpha-Proteobacteria detected between standard and extended preservation times. Lam and Cowen (2004) tested various methods for FISH sample storage. They reported probe-targeted cell loss during long term preservation (40 days) with formaldehyde preservation in suspension. Formaldehyde is monomer, and also is the smallest molecule among all aldehydes. Therefore, it may take longer for formaldehyde to form cross-links within cells, compared to paraformaldehyde and other aldehydes. Our results show that the oligonucleotide probes can penetrate the cross-linking structure formed by formaldehyde during the 4 or 5 days preservation in suspension. All samples in this study were filtered within 5 days after formaldehyde preservation, so this extended preservation time should not have affected the FISH results.

### 3.5.2 Comparisons of Bacterial Communities among Ballast Water Types

The % rod-shaped cells, % *Bacteria*, % Alpha-Proteobacteria, and % *Cytophaga-Flavobacteria* were not significantly different among the three ballast water types. However, the effective propagule pressure of each targeted phylogenetic group is total bacterial abundance measured by AO staining multiplied by the proportion of hybridized cells for each of the groups. The total bacterial abundance was more than 50% higher in NonMOE than TOE samples (Chapter 2, Table 2.2). Hence, the release of NonMOE ballast water into Canadian ports may have higher propagule pressure of each phylogenetic group than TOE or ICE.

### 3.5.3 Measured Bacterial Parameters: Comparisons between Ballast Water and Port Water Samples

Since only one port sample was collected from the GL, statistical analysis about the differences between port and ballast water samples at the GL site was not conducted. However, a sufficient number of ballast and port water samples is available to carry out robust statistical analyses between ballast and port water samples at the EC and WC sites.

A previous review(Glöckner et al. 1999) of the bacterioplankton compositions in lakes and oceans with FISH showed that the % *Bacteria* has a wide range among various aquatic environments, from 39% in the North Sea to 96% in the Antarctic Ocean in the surface (95% CI: 46 to 61%; $n = 26$). However, previous studies have reported lower %

*Bacteria* offshore than inshore (Bouvier and del Giorgio 2007; Garneau et al. 2006). Thus, the offshore sources of MOE ballast water samples could be one explanation for the lower % *Bacteria* in ballast (95% CI, 43 to 50%) than port (95% CI, 57 to 74%) water samples observed in this study. The difference in the % *Bacteria* between ballast and port water suggests that different bacterial communities could have been introduced by deballasting into Canadian waters.

The % *Archaea* was low, and they were found only in ballast and port water from the WC site. *Archaea* is one of the three domains of life along with the *Eukarya* and *Bacteria* (Woese 1990). *Crenarchaeota and Euryarchaeota* are two major groups of planktonic *Archaea* (Herndl et al. 2005). In previous studies, the relative abundance of *Crenarchaeota* was more abundant in mesopelagic and bathypelagic waters than in surface waters (Herndl et al. 2005), and *Euryarchaeota* appears to be a common element of coastal assemblages and surface waters (Pernthaler et al. 2002). However, in surface seawater, *Archaea* generally represents less than 2% of the total cells count (Bouvier and del Giorgio 2003). *Euryarchaeota* seasonally formed >30% of all cells in the surface picoplankton of the North Sea (Pernthaler et al. 2002). Seasonal blooms of *Euryarchaeota* were also observed during a long-term study in surface waters of the upper Santa Barbara Channel (Murray et al. 1999). *Archaea* can survive in extreme environments, such as hydrothermal vents and hot springs (DeLong 2003). However, the low % *Archaea* in all ballast water samples indicates that *Archaea* may survive but is not favored in the ballast tank environment during voyage. Alternatively, the lack of *Archaea* detection in ballast

water is that *Archaea* may not be present in the environment where ballast water was collected or exchanged.

Overall, 34% to 81% of bacteria in port water samples (mean ± SD: 68 ± 15, n = 59), and 20% to 78% of cells in ballast water samples (mean ± SD: 47 ± 14, n = 10) were detected by either EUB338 or ARCH915 probe in this study. Karner et al (2001) sampled monthly from September 1997 to December 1998 at the Hawai'i Ocean Time-series station in the North Pacific subtropical gyre, and they found that the sum of % *Bacteria* and % *Archaea* remained fairly constant in the surface waters through their sampling season, roughly 80% of the total cells.

The limitations of the FISH technique contribute to the sum of % *Bacteria* and % *Archaea* being lower than 100% of DAPI stained cells. For example, (1) If only small numbers of the targeted rRNA molecules are in a cell, the fluorescence intensity from the few binding probes may fall below detection limit (Amann et al. 1995). (2) Probe EUB338 is incapable of hybridizing with all taxa in the *Bacteria* domain, and excludes some taxa, such as *Planctomyces*. Kuypers et al (2003) reported at least 2 x $10^6$ cells $L^{-1}$ of anammox bacteria (members of the *Planctomyces*) in the Black Sea's suboxic layer, and so these bacteria can be abundant in some locations. Other limitations of this technique have been elaborated in reviews by Amann et al. (1995) and by Dorigo et al. (2005).

The relative abundance of cells that can respond to target oligonucleotide probes should represent the proportion of metabolically active bacteria in the water column. Faster growing or highly active cells have a greater rRNA content, and thus may bind proportionally more to probe molecules, resulting in a stronger fluorescence signal (Bouvier and del Giorgio 2003). This is supported by previous findings of a positive correlation between the universal 16S rRNA probe counts with the autoradiography of $^{3}$H-labeled amino acid uptake (Karner and Fuhrman 1997). If this is generally applicable, the response of target groups to FISH is an indicator of the physiological state of cells. The higher % *Bacteria* in port than in ballast water samples (Figure 3.2) might indicate that the *Bacteria* in port water samples are more active than in ballast water samples, in addition to a possible explanation that different bacterial communities were introduced by ballast water into Canadian waters.

The % Alpha-Proteobacteria ranged from 0% to 38% (mean ± SD: 17 ± 9%, n = 59) in ballast water samples, and from 0% to 30% (mean ± SD: 22 ± 9%, n = 10) in port water samples. Alpha-Proteobacteria are abundant free-living bacterioplankton in coastal and open-ocean habitats (DeLong et al. 1993). Although there was no significant difference in % Alpha-Proteobacteria between ballast and port water samples in both regions, our results show that Alpha-Proteobacteria is a common and abundant phylogenetic group in ballast water.

The % *Cytophaga-Flavobacteria* ranged from 0 to 44% (mean ± SD: 12 ± 9%, n = 59) in ballast water samples, and from 1 to 33% (mean ± SD: 24± 9%, n = 10) in port water

samples. *Cytophaga-Flavobacteria* can account for one third of all *Bacteria* in coastal marine waters (Bouvier and del Giorgio 2003), and ranged from 20 to 40% of the DAPI count on a transect from 49°S to 70°S during the austral summer (Simon et al. 1999). In our study, both the % rod-shaped cells and % *Cytophaga-Flavobacteria* showed the same pattern in the comparisons between ballast and port water samples at the EC and WC. Both % rod-shaped cells and % *Cytophaga-Flavobacteria* did not differ between ballast and port water samples at the WC. However, higher % rod-shaped cells and % *Cytophaga-Flavobacteria* was found in port than ballast water samples at EC, which suggests different bacterial communities may have been introduced to the EC.

*Vibrio* species are commonly found in marine environments and are readily cultured from seawater and marine animals (Lipp et al. 2002). Some representatives of *Vibrio* species, such as *Vibrio cholerae, V. vulnificus,V. harveyi*, and *V. parahaemolyticus* have been implicated in a variety of human diseases (Koenig 2009), and some of them, such as *V. alginolyticus*, *V. anguillarum*, and *V. penaeicida* are pathogens for a broad range of cultured marine organisms (Oliver et al. 1983). In our study, the % *Vibrio spp.* was low for both ballast and port water samples. *Vibrio spp.* was only detected from nine of 59 ballast water samples, and they ranged from 2 to 17% where they occurred. Similar results were reported in a previous study, which sampled 62 ballast tanks from 28 ships along the U.S. West Coast and East Coast (Burkholder et al. 2007). In their study, *Vibrio spp.* were detected in 16 of the 62 tanks, comprised 0-10% of the total bacterial abundances in the ballast tanks determined by quantitative PCR, but no toxigenic *V. cholerae* strains were detected in any tanks (Burkholder et al. 2007). However, toxigenic

*V. cholerae* O1 was recovered from ballast water from five out of 19 cargo ships docked in ports of the Gulf of Mexico by sample cultivation (McCarthy and Khambaty 1994). Ruiz et al (2000) studied 28 vessels arriving to Chesapeake Bay from foreign ports, and found *V. cholerae* in plankton samples from all studied ships, with toxigenic serotypes *V. cholerae* O1 and O139 detected in 93% of the ships. It should be noted that studies or pathogens commonly perform cultivation to enrich the number of pathogens within samples (McCarthy and Khambaty 1994). Without cultivation, some pathogens may be present, but in too low numbers to be detected, which might be the case of *V. cholerae* in Burkholder et al. (2007).

### 3.5.4 Measured Bacterial Communities Comparisons Among the Three Deballasting Locations

Ballast water from the Pacific is deballasted into the WC and ballast water from the Atlantic is deballasted into the EC and GL. However, using FISH, community composition differences between the Pacific and Atlantic Ocean sources were not detected. The % rod-shaped cell, % *Bacteria*, and % *Cytophaga-Flavobacteria* of combined ballast water samples did not differ among the three regions. The only difference among the three regions was in the % Alpha-Proteobacteria (Table 3. 4), which was significantly lower at the GL (the Atlantic source) than the EC (the Atlantic source) and WC (the Pacific source). Our results indicate that ocean sources are not the primary influencing factor for the bacterial community structure in ballast water deballasted in Canadian waters.

### 3.5.5 Microbial Community Structure and Physiochemical Parameters

Ballast water age and temperature influenced the bacterial community structure. With an increase in ballast water age, both the % *Bacteria* and the % Alpha-Proteobacteria decreased. The decreasing % *Bacteria* and % Alpha-Proteobacteria with the increase of ballast water age might indicate that the proportion of metabolically active *Bacteria* and Alpha-Proteobacteria in ballast water declined with ballast water age. Previous studies have reported that species richness and biomass of fish (Wonham et al. 2000), zooplankton (Gollasch et al. 2000), and phytoplankton (Burkholder et al. 2007) decreased as ballast water aged. Similar patterns have been reported that bacterial abundance declined with an increase in ballast water age during shipping voyage from Israel to the USA (Drake et al. 2002). The observed inverse relationships between % *Bacteria*, % Alpha-Proteobacteria and ballast water age in this study is possibly due to a number of biotic and abiotic reasons: (1) The DOM from the phyto- and zooplankton die-off during voyage could not be utilized by bacteria efficiently (Drake et al. 2002) (2) Bacteria are removed by micro-zooplankton grazing or virus lysis (Drake et al. 2002). (3) The reduction in dissolved oxygen (DO) or the release of toxic metal corrosion from the interior ballast tanks may also be a factor. None of the above reasons are mutually exclusive.

Temperature is often used as a predictor for bacterial activity, and many studies have shown positive relationships between bacterial growth rates and temperature (Pomeroy

and Wiebe 2001; Weston and Joye 2005). However, it is premature to relate limited number of physiochemical forcing to the bacterial community structure changes in ballast water, since ballast water tanks is a complex environment, and bacteria in ballast water experience series of survival selections (Carlton 1985). The negative relationship between the % *Cytophaga–Flavobacteria* in combined ballast water samples and the sample temperature (range from 4 to 28 °C) suggests that temperature control of bacterial community structure is influenced by other factors, such as substrate availability (Weston and Joye 2005).

## 3.6 Conclusion and Future Directions

Here we reported on the bacterial community structure in ballast and port water samples collected along the West and East Coast of Canada and on the Great Lakes. We compared the community structure among ballast water types, between ballast water and port water, and among three deballasting locations. In addition, physiochemical factors, which might regulate the bacterial communities in ballast water samples, were evaluated.

The three ballast water types defined by the Canada ballast water management regulations did not differ in the bacterial community structures introduced at each deballasting region. The bacterial communities in ballast water deballasted in Canadian waters show little regional patterns. The only detected difference among the three regions was that lower % Alpha-Proteobacteria in ballast water samples was deballasted into the Great Lakes than the East Coast and West Coast of Canada. However, different bacterial communities were

introduced to Canadian waters through ballast water. The % *Bacteria* in ballast water samples was significantly lower than that in port water samples at the EC and WC. In addition, lower % rod-shaped cells and % *Cytophaga-Flavobacteria* in ballast water were deballasted into the ports at the EC. Ballast water age and temperature influenced bacterial community structures in ballast water tanks. An inverse relationship was observed between % *Cytophaga-Flavobacteria* in ballast water samples and temperature. The results presented here suggest that there are regulatory factors other than temperature influencing bacterial community structure in ballast water. With an increase in ballast water age, both the % *Bacteria* and the % Alpha-Proteobacteria in ballast water decreased. The negative relationships observed between ballast water age and bacterial communities imply controlling ballast water age could have a possible ballast water management application. We would suggest that ships carry out MOE at the beginning of shipping voyage, instead of at the end of voyage to increase the ballast water age.

Here the introduced bacterial communities from ballast water into Canadian waters were reported. However, the fate of bacteria after discharged from ballast tanks, and the regional susceptibility to invasion are poorly understood. Further study of the adaptation of introduced bacterial communities to the environmental conditions in the receiving waters and interactions between introduced bacterial communities and native communities are needed to understand the survival and reproduction of introduced bacteria in new environment.

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Table 3.1 Oligonucleotide probes, sequences, target bacterial groups, and percentage of formamide required for hybridization.

| Oligonucleotide Probe | Sequence (5'-3') | Specificity | Formamide in Hybridization Buffer | NaCl Required in Washing Buffer | Reference |
|---|---|---|---|---|---|
| EUB338 | GCTGCCTCCCGTAGGAGT | Most *Bacteria*; not *Planctomyces* | 35% | 70 mM | Amann et al. 1990 |
| CF319a | TGGTCCGTGTCTCAGTAC | *Cytophaga-Flavobacteria* | 35% | 70 mM | Manz et al. 1996 |
| ALF968 | GGTAAGGTTCTGCGCGTT | Alpha-Proteobacteria | 20% | 215 mM | Neef et al. 1999 |
| ARCH915 | GTGCTCCCCCGCCAATTCCT | *Archaea* | 35% | 70 mM | Stahl et al. 1991 |
| GV | AGGCCACAACCTCCAAGTAG | *Vibrio spp.* | 30 % | 112mM | Eilers et al. 2000; Giuliano et al. 1999 |
| Eco16S07C | ACTTTACTCCCTTCCTC | *Escherichia coli, Shigella, P. aeruginosa* | 10% | 450mM | Stender et al. 2001 |
| NON338 | ACTCCTACGGGAGGCAGC | Negative control probe; complementary to EUB338 | 35% | 70 mM | Wallner et al. 1993 |

Table 3.2 Percent rod-shaped cells among sample types, in each sampling region.

| Sampling Location | Sample Types[1] | n | Minimum | Maximum | Mean | SD | CV | 95% CI | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | LL | UL |
| East Coast | port | 5 | 22.1 | 30.3 | 27.6 [a] | 3.3 | 11.8 | 23.5 | 31.6 |
| | TOE | 19 | 10.8 | 32.9 | 18.9 [b] | 5.8 | 30.4 | 16.2 | 21.7 |
| | ICE | 4 | 17.0 | 25.2 | 20.4 [ab] | 3.7 | 18.0 | 14.6 | 26.3 |
| | NonMOE | 0 | - | - | - | - | - | - | - |
| Great Lakes | port | 1 | - | - | 14.6 [na] | - | - | - | - |
| | TOE | 8 | 10.9 | 27.8 | 18.6 [a] | 5.7 | 30.6 | 13.9 | 23.4 |
| | ICE | 3 | 14.3 | 17.1 | 15.4 [a] | 1.5 | 9.5 | 11.8 | 19.0 |
| | NonMOE | 1 | - | - | 11.3 [na] | - | - | - | - |
| West Coast | port | 3 | 15.8 | 23.0 | 20.5 [a] | 4.0 | 19.8 | 10.4 | 30.6 |
| | TOE | 14 | 11.2 | 36.9 | 20.4 [a] | 8.5 | 41.5 | 15.5 | 25.3 |
| | ICE | 8 | 9.9 | 35.0 | 17.6 [a] | 8.6 | 48.8 | 11.0 | 24.2 |
| | NonMOE | 6 | 14.2 | 22.0 | 16.9 [a] | 3.1 | 18.3 | 13.1 | 20.8 |

**Notes:**

"1": Port water samples (port), trans-oceanic exchanged ballast water (TOE), intra-coastal exchanged ballast water (ICE), and without mid-ocean exchange ballast water (NonMOE).

Analyses of Variances were used to determine the overall differences in % rod-shaped cells among sample types at each location. Tukey 95% simultaneous confidence intervals were used to determine the differences among sample types.

"a", "b", "na" and "ab": Significant differences among mean values of ballast water types at each locations and each year are indicated by superscript codes: mean with superscript "a" has significantly larger value than that with superscript "b"; "ab" indicate there are no significant difference between means with "a" and "ab", or between means with "b" and "ab"; means with same superscript are not significant different; mean with "na" was not compared because of the limited sample numbers.

Table 3.3 Proportion of cells detected as *Bacteria* and *Archaea* ((probe positive/DAPI positive) x 100) among ballast water types, in each sampling region.

| Sampling Location | Ballast Water Types[1] | n | % *Bacteria* | | | | | % *Archaea* | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Minimum | Maximum | Mean | SD | CV | Minimum | Maximum | Mean | SD | CV |
| East Coast | TOE | 18 | 20 | 66 | 42 | 15 | 36 | 0 | 1.3 | 0.1 | 0.3 | 268 |
| | ICE | 4 | 48 | 64 | 56 | 9 | 15 | 0 | 1.9 | 0.6 | 0.9 | 161 |
| | NonMOE | 0 | - | - | - | - | - | - | - | - | - | - |
| Great Lakes | TOE | 8 | 28 | 78 | 48 | 14 | 30 | 0 | 0.9 | 0.3 | 0.3 | 163 |
| | ICE | 3 | 39 | 49 | 44 | 7 | 16 | 0 | 0.0 | 0 | 0.0 | 141 |
| | NonMOE | 1 | - | - | 53 | - | - | - | - | 0.3 | - | - |
| West Coast | TOE | 13 | 21 | 64 | 45 | 12 | 27 | 0 | 5.9 | 2 | 1.8 | 101 |
| | ICE | 8 | 28 | 73 | 51 | 13 | 25 | 0 | 3.4 | 1 | 1.2 | 132 |
| | NonMOE | 4 | 43 | 71 | 54 | 12 | 22 | 0 | 4.0 | 3 | 2.0 | 75 |

**Notes:**

"1": Trans-oceanic exchanged ballast water (TOE), intra-coastal exchanged ballast water (ICE), and without mid-ocean exchange ballast water (Non-MOE).

Table 3.4 Proportion of cells detected as Alpha-Proteobacteria, and *Cytophaga-Flavobacteria* ((probe positive/DAPI positive) x 100) among sample types, in each sampling region.

| Sampling Location | Sample Category[1] | n | % Alpha – Proteobacteria | | | | | % *Cytophaga - Flavobacteria* | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Minimum | Maximum | Mean | SD | CV | Minimum | Maximum | Mean | SD | CV |
| East Coast | port | 5 | 18 | 28 | 23 [ab] | 4 | 18 | 22 | 33 | 29 [a] | 4 | 14 |
| | TOE | 18 | 0 | 38 | 16 [b] | 11 | 67 | 0 | 36 | 10 [b] | 9 | 97 |
| | ICE | 4 | 23 | 36 | 28 [a] | 6 | 21 | 3 | 25 | 13 [b] | 11 | 83 |
| | NonMOE | 0 | - | - | - | - | - | - | - | - | - | - |
| Great Lakes | port | 1 | - | - | 0 [na] | - | - | - | - | 1 [na] | - | - |
| | TOE | 8 | 4 | 34 | 11 [a] | 10 | 85 | 2 | 31 | 11 [a] | 9 | 82 |
| | ICE | 3 | 3 | 7 | 5 [a] | 3 | 52 | 4 | 7 | 5 [a] | 2 | 43 |
| | NonMOE | 1 | - | - | 6 [na] | - | - | - | - | 16 [na] | - | - |
| West Coast | port | 4 | 23 | 30 | 26 [a] | 3 | 11 | 21 | 27 | 24 [a] | 3 | 11 |
| | TOE | 13 | 7 | 30 | 18 [a] | 6 | 34 | 2 | 26 | 12 [a] | 8 | 63 |
| | ICE | 8 | 13 | 28 | 19 [a] | 5 | 25 | 2 | 44 | 15 [a] | 13 | 88 |
| | NonMOE | 4 | 15 | 24 | 20 [a] | 4 | 20 | 5 | 30 | 17 [a] | 11 | 65 |

**Notes:**

"1": Port water samples (port), trans-oceanic exchanged ballast water (TOE), intra-coastal exchanged ballast water (ICE), and without mid-ocean exchange ballast water (NonMOE).

Analyses of Variances were used to determine the overall differences in % Alpha-Proteobacteria and % *Cytophaga-Flavobacteria* among sample types at each location. Tukey 95% simultaneous confidence intervals were used to determine the differences among sample types.

"a", "b", "na" and "ab": Significant differences among mean values of ballast water types at each locations and each year are indicated by superscript codes: mean with superscript "a" has significantly larger value than that with superscript "b"; "ab" indicate there are no significant difference between means with "a" and "ab", or between means with "b" and "ab"; means with same superscript are not significant different; mean with "na" was not compared because of the limited sample numbers.

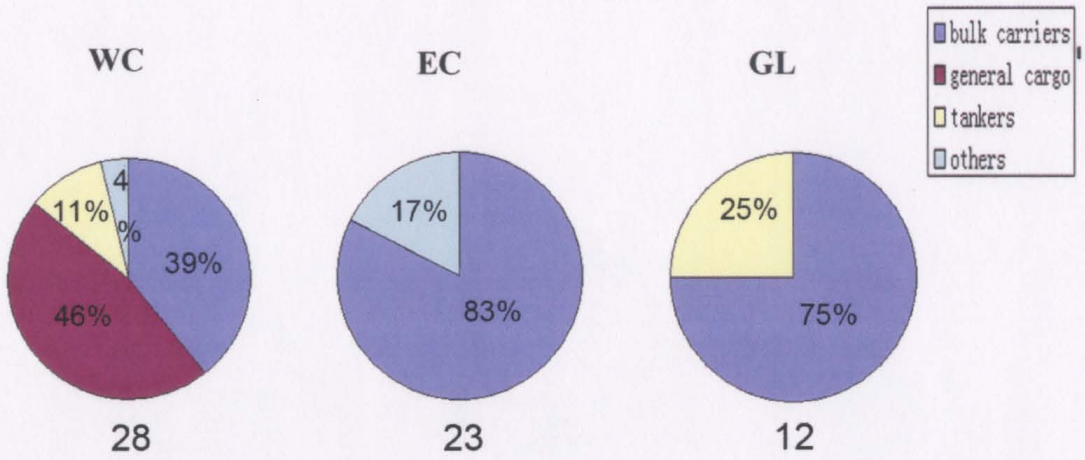


Figure 3.1 Ballast water samples by vessel types in the three regions. The number of ballast water samples collected (below) and the percentages by vessel types (bulk carriers, general cargo, tankers and others) at the West Coast (WC) and East Coast of Canada (EC), and the Great Lakes (GL) sites. Data have been collected from ballast water management forms.

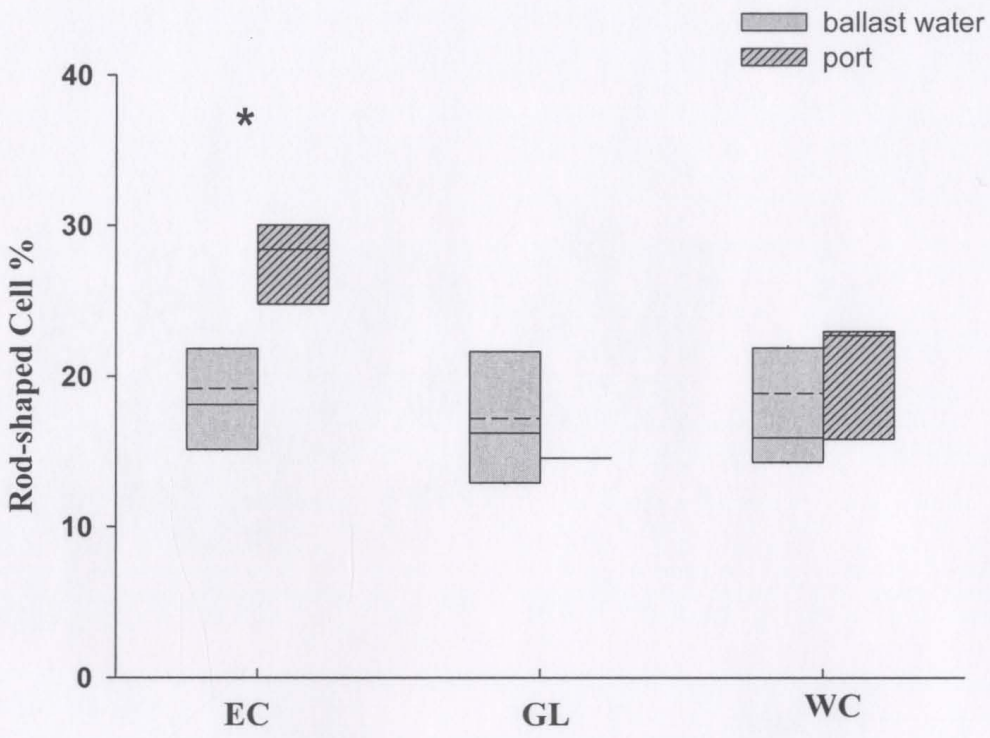


Figure 3.2 Percent rod-shaped cell in ballast (grey) and port (striped) water samples for the West Coast (WC) and East Coast of Canada (EC), and the Great Lakes (GL). The lower and upper boundaries of each box are the 25th and 75th percentile, respectively. The dashed and solid lines within the boxes are the mean and the median, respectively. "*" indicates bacterial abundance is significantly higher in port than in ballast water samples (ANOVA, $p < 0.05$).

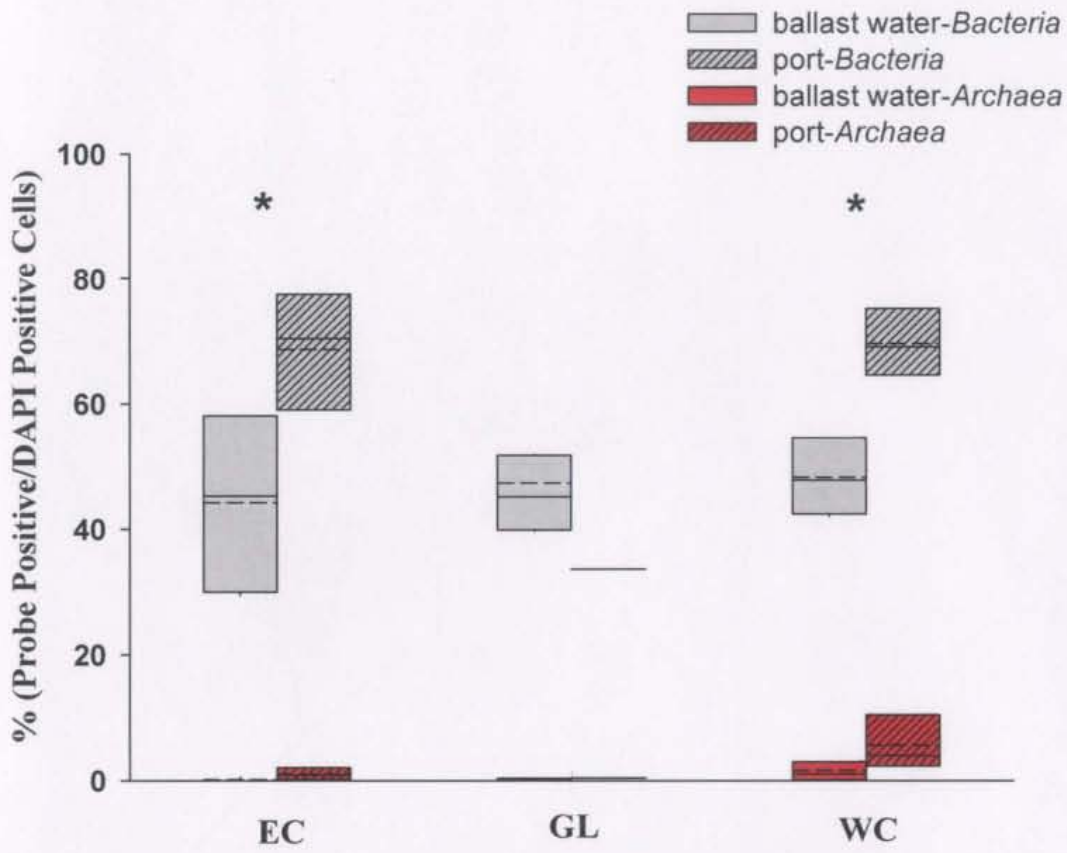


Figure 3.3 The % *Bacteria* (grey) and % *Archaea* (red) in ballast water samples (non-pattern) and port water samples (striped) in the West Coast (WC) and East Coast of Canada (EC), and the Great Lakes (GL) sites. The lower and upper boundaries of each box are the 25th and 75th percentile, respectively. The dashed and solid lines within the boxes are the mean and the median, respectively. "*" indicates that the proportion of cells detected as *Bacteria* was significantly higher in port than in ballast water samples (ANOVA, $p < 0.05$). Too few port samples were collected in the GL to permit statistical analysis.

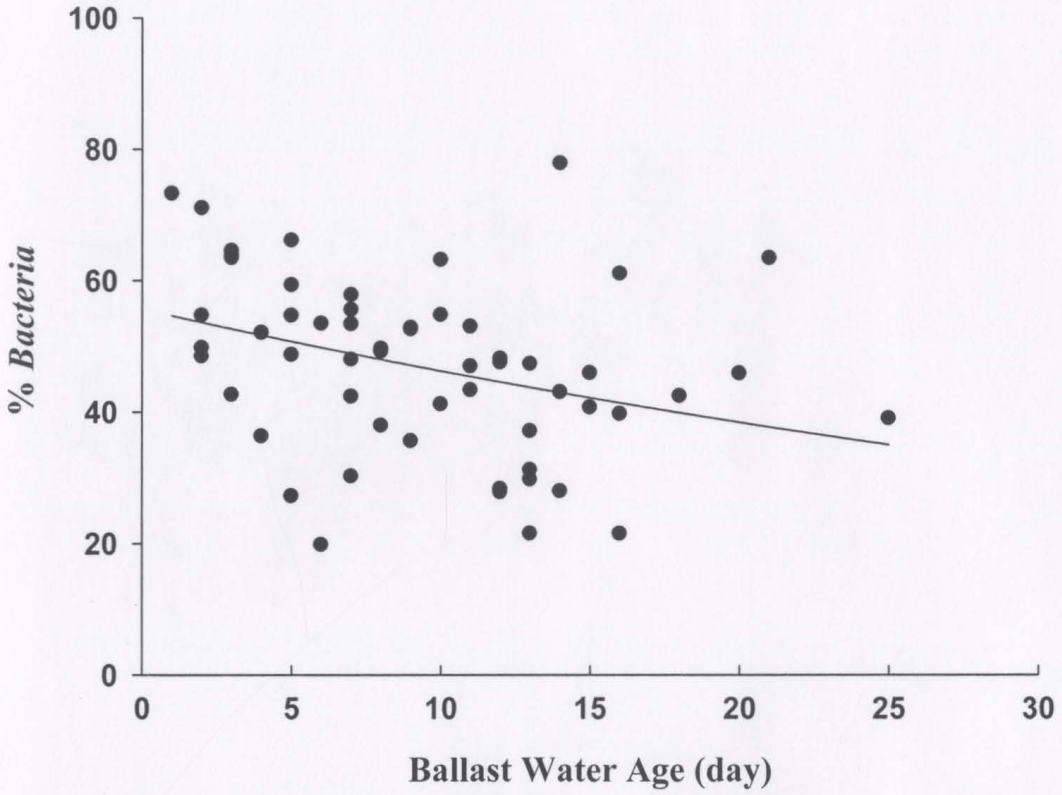


Figure 3.4 Relationship between the % *Bacteria* and ballast water age (% *Bacteria* = 55.63 $e^{-0.02 \times \text{Age (day)}}$, $r^2 = 0.10$, n = 59, P = 0.014).

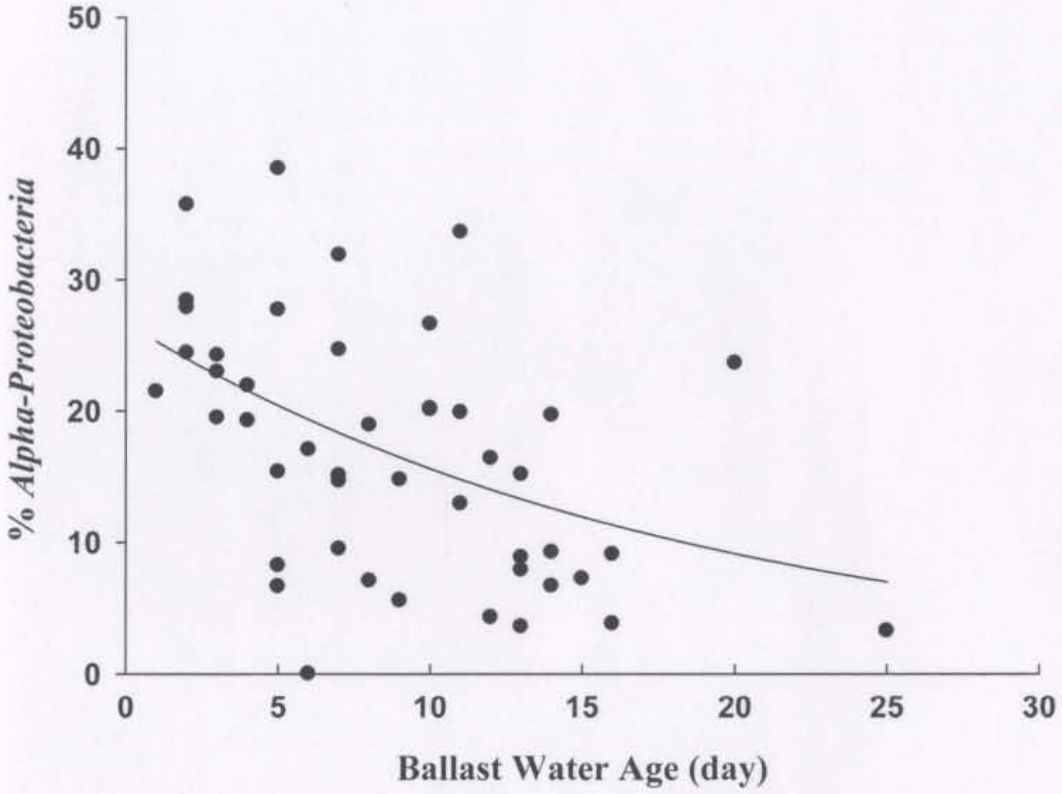


Figure 3.5 Relationship between the % Alpha-Proteobacteria and ballast water age (% Alpha-Proteobacteria = $26.70\, e^{-0.05 \times \text{Age (day)}}$, $r^2 = 0.24$, $n = 59$, $P < 0.001$).

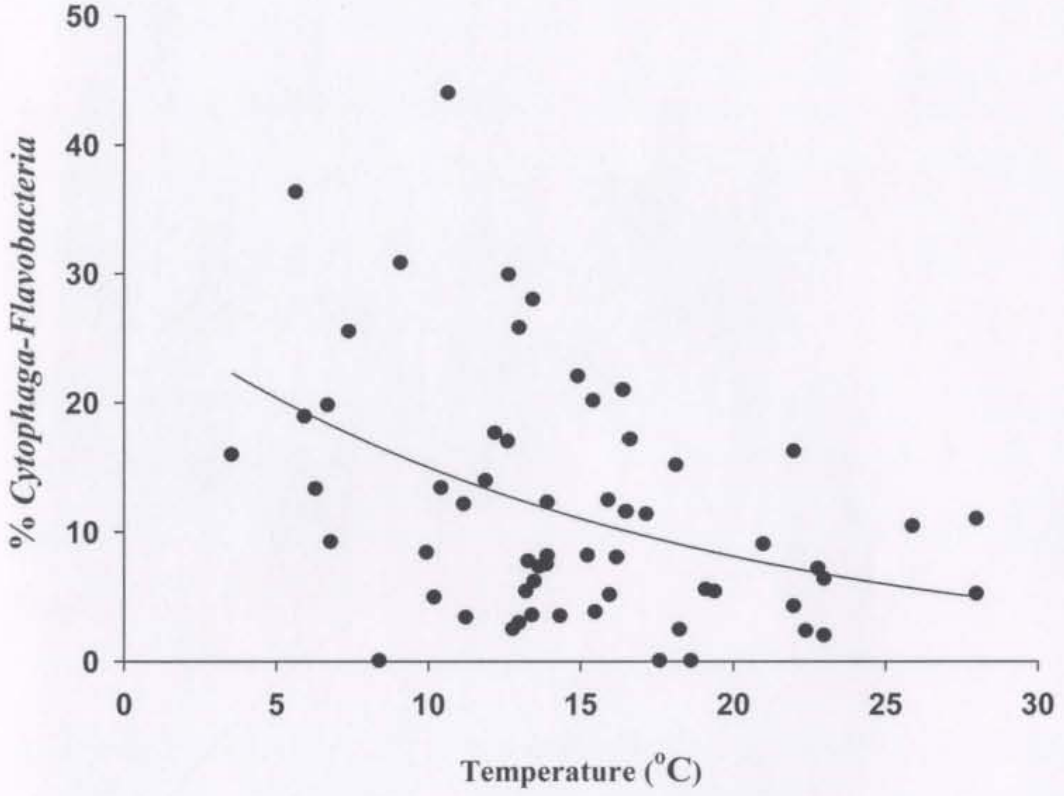


Figure 3.6 Relationship between the % *Cytophaga-Flavobacteria* and ballast water age (% *Cytophaga-Flavobacteria* = 27.72 $e^{-0.06 \times \text{Temp (°C)}}$, $r^2 = 0.16$, n = 59, P = 0.002).

# Chapter 4 Summary

## 4.1 Synopsis of Manuscript Chapters

Over 80% of the world's cargo is mobilized internationally by sea-going ships. Ballast water has been recognized as one of the primary vectors for the global transport of vegetative and resting stages of aquatic microorganisms (Carlton and Geller 1993; Ruiz et al. 2000). Previous studies have shown that ships can globally distribute pathogens, such as *Vibrio cholerae* and *Escherichia coli* (Ruiz et al. 2000). Successful aquatic invasion of non-indigenous species can cause unwanted economic (Pimentel et al. 2005), ecological (Carlton et al. 1990; Mills et al. 1993) and human health impacts (McCarthy and Khambaty 1994).

Heterotrophic prokaryotes (hereafter bacteria) are small, unicellular organisms that are ubiquitous in the worlds' oceans (Sherr and Sherr 2000). Bacterial abundance has been reported ranging from $10^7$ to $10^{10}$ cell $L^{-1}$ in aquatic environment (Whitman et al. 1998). Thus, bacteria are ready to be entrained into ballast tanks with sea water uptake and to be released at receiving ports when ballast water is deballasted. Positive correlations have been shown between propagule pressure (the number of individuals introduced into a given environment) and the number of successful species that can be established in their new environment (Lockwood et al. 2005). The abundance of microorganisms in ballast waters has been considered as a proxy for propagule pressure (Drake and Lodge 2007). Therefore, a study about bacterial abundance in ballast water will benefit future risk assessment modeling about bacterial invasion through ballast water.

Bacteria which are redistributed in ballast water, could impact both the bacterial community composition and the ecological function of receiving waters. Bacteria have a central role in mediating biogeochemical processes of the ocean, including the cycling of organic carbon, nitrogen, sulfur, phosphorus and other organic and inorganic elements (Fenchel et al. 1998; Newman and Banfield 2002). However, specific bacterial phylotypes differ in their contribution to the marine biogeochemical processes (Fuhrman 2009). Thus, studying the abundance and activity of different phylogenetic groups within bacterial communities is the first step in understanding the potential impacts of ballast water introduced bacteria on the ecological function of receiving regions.

To reduce the impacts of ballast water-mediated organisms, the International Maritime Organization (IMO) established mid-ocean ballast water exchange (MOE) guidelines in 1991. In Canada, ballast water from the arrival of all international ships falls into three categories: trans-oceanic exchanged (TOE), intra-coastal exchanged (ICE) and without mid-ocean exchange (NonMOE), according to whether MOE is conducted and where MOE is conducted (Transport Canada 2006). Hence, it is essential that the effects of these different ballast water operations on bacterial communities be studied.

Chapter 2 presented the results of the spatial patterns in bacterial abundance and cell volume in ballast and port water samples collected along the West Coast (WC) and East Coast (EC) of Canada and from the Great Lakes (GL) in 2007 and 2008. The results here showed that higher bacterial abundance and hence potentially greater propagule pressure were introduced

by unexchanged ballast water than ballast water exchanged at sea. Although lower bacterial abundance was observed in ballast water compared to port water in this study, the bacterial abundance in ballast water was still as high as $10^7$ to $10^9$ cells $L^{-1}$. In this study, the bacterial abundance and cell volume showed inconsistent spatial distribution in ballast water samples among the three deballasting locations. For example, higher bacterial abundance in ballast water was found at the EC, compared to the WC and the GL in 2008. However, there was no difference in bacterial abundance of ballast water samples among the three regions in 2007. Ballast water age and temperature were the only influential factors detected for bacterial abundance and cell volume in ballast water samples. A negative relationship was found between the bacterial abundance of ICE samples and ballast water age, while a positive relationship was found between cell volumes of 2007 TOE samples and temperature. However, the lack of relationships between the measured physiochemical parameters and bacterial variables in all three ballast water types during both years indicates there are other regulatory factors influencing the bacteria in ballast water.

Chapter 3 presented the results of a study on the % rod-shaped cells and the bacterial community structure in ballast and port water at the EC, WC and GL during 2007. Bacterial community structure was characterized using fluorescence *in situ* hybridization (FISH), and was related to a number of concurrently-measured physiochemical parameters (ballast water age, temperature, pH, and salinity). Although the ballast water deballasted into Canadian jurisdiction may experience three different operations, there was no difference detected in bacterial community structure among the ballast water types in each deballasting region. Different bacterial communities from those in Canadian waters were introduced by ballast

water. The % *Bacteria* in ballast water samples was significantly lower than that in port water samples at the EC and WC, and both the % rod-shaped cells and *% Cytophaga-Flavobacteria* were lower in ballast than in port water samples at the EC. The bacterial communities in ballast water deballasted in Canadian waters show little spatial patterns. Lower % Alpha-Proteobacteria in ballast water samples deballasted into the GL than the EC and WC was the only detected difference in bacterial communities among the three regions. In this study, *Vibrio spp.* was only detected from nine of 59 ballast water samples, and % *Vibrio spp.* ranged from 2 to 17% where they occurred. Although the fecal contamination indicator bacterium *E. coli* was not detected, we cannot exclude its presence at abundance below the detection limit of FISH. Ballast water age and temperature were the only influential factors detected for bacterial community structure in ballast water tanks. With an increase of ballast water age, both the % *Bacteria* and the % Alpha-Proteobacteria in ballast water samples decreased. A negative relationship between the % *Cytophaga-Flavobacteria* in combined ballast water samples and the sample temperature was observed in this study. This implies there are regulatory factors other than temperature regulating bacterial community structure in ballast water.

## 4.2 Overall Significance

The results of our study contribute to the current understanding of bacterial abundance and phylogenetic diversity of the bacterial community in ballast water. To my knowledge, this is the first study that examined both bacterial abundance and community structure on a large spatial and seasonal scale. Prior studies about bacteria in ballast water have been limited to

bacterial abundance (Drake et al. 2001, 2002; Ruiz et al. 2000) and the presence of pathogens (Ruiz et al. 2000). Moreover, no prior studies have collected ballast water samples from commercial ships on such a large scale. Large number of ballast water samples has been collected from military vessels in the United States (Burkholder and Glibert, 2006). However, it is the commercial ships that globally transport ballast water in massive volumes and follow the IMO regulations, which makes this study relevant. It provides an opportunity to estimate the role of ballast water globally redistributing bacteria, and how IMO regulations can affect the bacterial community in ballast water.

This study has shown that both the total bacterial abundance and the proportion of physiologically active cells decreased with the increase of ballast water age. Similar results were reported in previous studies of species richness and the biomass of fish (Wonham et al. 2000), zooplankton (Gollasch et al. 2000), and phytoplankton (Burkholder et al. 2007) decreased as ballast water aged. The negative relationships observed between ballast water age and the number of organisms present in ballast water imply that increasing the age of ballast water could have a management application for ballast water introduced invasions. we would suggest that ships carry out MOE at the beginning of shipping voyage, instead of at the end of voyage to increase the ballast water age.

## 4.3 Future Directions

Before a species can successfully invade a new environment and become established, it must first overcome a series of barriers. MacIsaac et al (2007) summarized these barriers as

biogeographic filter, physiological filter, and biotic filter. Our study described how bacteria may survive the biogeographical barrier by "hitching a ride" in ballast water and how they may affect the bacterial abundance and community in introduced environment. Further studies concerning the adaptation of introduced bacterial communities to new environmental conditions in receiving waters, as well as the interactions between introduced bacterial community and native community are needed to understanding how bacteria are capable of surviving these barriers. This in turn may allow the development of predictive and intention tools.

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